REMARKS

Claims 1, 2 and 20-25 are pending. Claims 3-17 were cancelled by preliminary amendment, and claims 18, 19 and 26 were found by the Examiner to be drawn to separate inventions following a three-way restriction. Group I, drawn to claims 1, 2 and 20-25 was elected and claims 18, 19 and 26 were cancelled without prejudice to permit immediate issue of claims 1, 2 and 20-25. Following payment of the issue fee, the application was withdrawn from issue in view of art previously of record in the application and the present Office Action issued. That art is addressed below.

Rejection under 35 U.S.C. §102:

The Office Action states that claims 1, 2 and 20-25 are rejected under 35 U.S.C. §102(b) as being anticipated by Ohkuma et al. (U.S. 5,541,181). The Office Action states:

> The claims read on the compound made by the microorganism, strain M990-6, identified as being a species of *Micromonospora*. The reference depicts the structure of the compound isolated incorrectly. However, the later published correction by Igarashi et al. (in J. Antibiot. 58 (5): 350-352 (2005)) revised the structure of the reference.
>
> This anticipation rejection is made on the compound produced by the same microorganism and has the same NMR spectrum as in the prior art (with the difference of solvent peaks). Therefore, the compound claimed was first produced by Ohkuma et al. and the claims read thereon.

The premise of the rejection appears to be that a prior art reference's (Ohkuma et al.) disclosure of a clearly non-anticipatory compound is rendered inherently anticipatory by a non-prior art publication (Igarashi et al., by a group of investigators separate from Ohkuma et al.) which proposes a "revised" structure. Applicants respectfully, but strongly disagree.

Not only is there no evidence that the authors of the Ohkuma et al. reference erred in assigning the structure of the compound reported in that reference, but there is also no evidence that the compound that the Igarashi et al. authors tested is the same as that

produced by *Micromonospora* strain M990-6. As such, the Ohkuma et al. reference does not either literally or inherently disclose the presently claimed compound.

Under the law, "a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed Cir. 1987). Additional references can be used to show an inherent, but not specifically described, characteristic of the thing taught by the primary reference. Such evidence, however, "must make clear that the missing descriptive matter is ***necessarily present*** in the thing described in the reference, and that it would be so recognized by persons of ordinary skill." *Continental Can Co. v. Monsanto Co.*, 948 F.2d 1264, 1268 (Fed. Cir. 1991); emphasis added. This doctrine has been consistently upheld by the Court of Appeal for the Federal Circuit, most recently in *Atofina* v. *Great Lakes Chemical Corporation*, 441 F.3d 991, 1000 (Fed. Cir. 2006), wherein Lourie J. stated "... anticipation by inherent disclosure is appropriate ***only*** when the reference discloses prior art that ***must necessarily include*** the unstated limitation ...". (It is noted that failure of those skilled in the art to contemporaneously recognize an inherent property, function or ingredient of a prior art reference does not preclude a finding of anticipation. *Schering Corp. v. Geneva Pharm. Inc.*, 339 F.3d 1373, 1377 (Fed. Cir. 2003).) Given this background in the law and the teachings of the Ohkuma et al. and Igarashi et al. references as discussed in detail below, Applicants submit that the Igarashi et al. reference does not in any way establish that the actual structure of BU-4664L produced by *Micromonospora* species strain M990-6 is the structure determined for the compound isolated from TP-A0860 by Igarashi et al. That is, the structure described by Igarashi et al. is not **necessarily present** in the compound described by Ohkuma et al., as is required for the Ohkuma et al. reference to anticipate, expressly or inherently, the claimed invention.

The microorganism used by Igarashi et al. is not the same as that used by Ohkuma et al.

First, Applicants would like to address a statement in the Office Action that appears to reflect an erroneous conclusion. The Office Action states that "the anticipation rejection is made on the compound produced by the *same microorganism*" (emphasis added). This is incorrect. The compound tested by Igarashi et al. was produced by the strain TP-A0860, isolated from a soil sample taken in Osawano, Toyama, Japan and characterized as a *Micromonospora* strain "based on the taxonomic study." In contrast, *Micromonospora* strain M990-6, from which compound BU-4664L was isolated by Ohkuma et al., was isolated from a soil sample taken in Colombo, Sri Lanka, approximately 4,500 kilometers distant from the site of origin of TP-A0860. Thus, while both sources of the compounds tested by the respective parties may be of the genus *Micromonospora*, neither is speciated nor is there any evidence that they are identical. There are a multitude of different *Micromonospora* species and within each species there are numerous different strains, and different strains of the same species are not by any means identical; **even the Igarashi et al. authors do not state or imply that TP-A0860 and M990-6 are the same microorganism.** As such, **there is no basis for concluding** that the BU-4664L compound produced by *Micromonospora* species strain M990-6 and isolated by Okhuma et al. is **necessarily** the secondary metabolite produced by TP-A0860 and isolated by Igarashi et al. , as required under the law on inherency. *Applicants further wish to emphasize that the cited Igarashi et al. paper was* ***not*** *published by a group with any connection to the group of investigators which generated the Ohkuma et al. reference – that is, the Igarashi et al. paper is* ***by no means*** *a retraction of the structural determination reported by Ohkuma et al.*

There is no expectation that different *Micromonospora* species strains would produce the same secondary metabolites

Second, a person of skill in the art would not hold any *a priori* expectation that the *Micromonospora* strain isolated by Ohkuma et al. (*i.e.* M990-6) and the *Micromonospora* strain isolated by Igarashi et al. (*i.e.* TP-A0860) would produce the

same secondary metabolites (especially when the different microorganisms are grown under different culture conditions – as is factually evident on comparison between the description provided in US Patent 5,541,181 and that provided in Igarashi et al. (2005) and the reference cited therein, namely Igarashi, Y. et al. (2002) "Pteridic acids A and B, novel plant growth promoters with auxin-like activity from *Streptomyces hygroscopicus* TP-A0451" J. Antibiotic. 55(8): 764-767, a copy of which is herein attached as **Exhibit A**. Conversely, given that persons of skill in the art accept, as fact, that members of the genus of *Micromonospora*, although taxonomically related, differ from one another genetically and in metabolic capabilities. There is therefore the very real expectation that different members of the genus *Micromonospora* would produce <u>different</u> secondary metabolites. In his accompanying Rule 132 Declaration, Dr. Julian Davies provides evidence as to the genetic and biochemical diversity and complexity with regards to microorganisms that comprise the microbial genus *Micromonospora* (paragraph 8 of the Davies Declaration), as well as providing an explanation as to the basis for why a person of skill in the art would hold no expectation that the microorganisms comprising different strains of *Micromonospora* sp. would produce the same secondary metabolites (paragraphs 8 and 13 of the Davies Declaration). Okhuma et al. isolated a distinct microorganism, a fact that Dr. Davies has corroborated in his Declaration (paragraphs 10, 11 and 12 of the Davies Declaration), that forms one member of a tremendously large genus that is known to produce a vast number of different secondary metabolites. As such, there is every reason to believe that Ohkuma et al. properly determined the structure of the compound that they isolated from their fermentation culture of their *Micromonospora* sp. strain M990-6 (*i.e.* compound BU-4664L), and that Igarashi et al. properly determined the structure of a different compound (*i.e.* one which they isolated from their fermentation culture of their *Micromonospora* sp. strain TP-A0860). The error, then, if any, would not be in Ohkuma et al.'s assignment of a structure to the compound that Ohkuma et al. isolated from strain M990-6, but in Igarashi et al.'s attempt to force the structure of their strain TP-A0860 derived molecule onto that of BU-4664L.

The Ohkuma et al. investigators confirmed their basic structural determination on multiple related molecules

Third, Applicants submit that Ohkuma et al. investigators separately determined the structure of **four** separate, yet related, compounds: BU-4664L, an acetylated derivative, an alkylated derivative (generated by methylation of BU-4664L), and a derivative lacking the farnesyl group (made from methanolysis of the alkylated derivative). *Separate* NMR analyses were conducted in relation to *each* of the four molecules and the NMR data for each molecule is specific to the particular molecule for which the data was analyzed. A structural determination was made for *each* of the four molecules. Applicants submit that it is highly unlikely that these researchers generated incongruous results on four separate occasions, and/or on each separate occasion misinterpreted their own data and drew an incorrect conclusion as to the proper structure of the compound they had isolated.

More specifically, regarding the placement of the BU-4664L farnesyl group, it is to be noted that the positioning by Ohkuma et al. of the farnesyl group was **not only** based upon the structural determination of the "parent" molecule alone, but rather was confirmed both by the independent structural determinations of the acetylated and the alkylated (trimethyl) derivatives and the independent structural determination of the non-farnesylated derivative (Compound-I), as based upon the NMR spectral data for each derivative . Thus, there is no reason to doubt Ohkuma et al.'s conclusion regarding the structure of BU-4664L.

Applicants note that Ohkuma et al. are quite definitive in: (a) characterizing their data and (b) drawing conclusions from their interpretation of their data. The wording provided in the reference with respect to the determination of the structure of each molecule is unequivocal. As indicated by the underscored words and phrases starting at line 46 of column 13 to line 25 of column 15 of the Ohkuma et al. reference copied below, the inventors properly determined in accordance with scientifically acceptable and valid methods the structure of the molecule that they isolated from a fermentation broth of *Micromonospora* sp. M990-6. This conclusion is supported by the accompanying

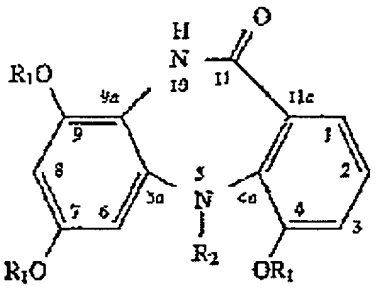
Rule 132 Declaration of Professor Lester A. Mitscher (paragraph 8 of the Mitscher Declaration). Relevant text from the Ohkuma et al. reference includes the following:

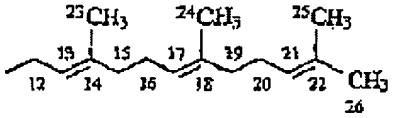
> The molecular formula of 1 was established as C_{28} H_{34} N_2 O_4 from the FAB-MS (positive, m/z 462(M^+) and negative, m/z 461(M-H)$^-$) and the microanalysis. Its ^{1}H and ^{13} C-NMR spectral data (FIGS. 2 and 3; Tables 6 and 7) revealed the presence of 1,2,3-trisubstituted (A ring) and 1,2,3,5-tetrasubstituted (B ring) benzene rings, three phenolic hydroxyl and one amido groups together with signals assignable to a farnesyl residue [...]. The farnesyl residue was linked to a nitrogen atom in view of the NMR chemical shifts of its terminal methylene [δ_H 4.39 (d, J=6.0 Hz) and δ_c 47.4(t)] [...]. The stereochemistry within the farnesyl residue was established as all (*E*) based on the ^{13}C-NMR chemical shifts [...] of the vinyl methyl groups (23-CH_3: δ 16.1, 24-CH_3: δ 15.7 and 25-CH_3: δ 17.4) except for the 26-CH_3 group (δ_H 25.3) resonated at lower field. In the ^{1}H-NMR spectrum, the remaining portion of the structure ($C_{13}H_9N_2O_4$) showed three contiguous protons (δ_H 6,89, 7.05 and 7.22) and a pair of meta coupling protons (δ_H 6.46 and 6.50, d, J=2.6 Hz) due to A and B benzene rings, respectively, together with one amido (δ_H 6.72) and three phenolic hydroxyl groups (δ_H 9.03, 9.94 and 10.03). The ^{13}C-NMR spectral data (FIG. 3 and Table 7) confirmed the presence of a farnesyl residue [...] and indicated the presence of twelve carbons including three oxygen bearing aromatic carbons (δ_H 145.4, 147.5 and 152.9) and one amido carbon (δ_H 167.5) due to the remaining portion.

Acetylation of 1 in pyridine gave the triacetate (2) which showed a pseudomolecular ion at m/z 589(M+H)$^+$ in the FAB-MS spectrum. The ^{1}H NMR spectrum of 2 revealed the presence of three acetyl groups (δ 2.23, 2.37 and 2.39) and showed downfield shifts for meta coupling protons (δ 6.86 and 7.08) of B ring and one of three contiguous protons (δ 7.50) when compared with those of 1 (6-H, δ 6.15; 8-H, δ 6.17 and 3-H, δ 6.70, respectively), supporting that three phenolic hydroxyl groups locate at ortho position of these protons in 1. Methylation of 1 with diazomethane in benzene-methanol mixture yielded the trimethyl derivative which showed the molecular ion at m/z 504 (M^+) together with a fragment ion at m/z 300 (M^+-farnesyl, base peak) in the EI-MS spectrum. The ^{1}H NMR spectrum of 3 (Table 6) is closely related to that of 1 except for three methoxy groups (δ 3.68, 3.84 and 3.88). In the ^{13}C-^{1}H long range COSY experiment of 3, the correlations were observed between a proton (H-1, δ 7.22) of A ring and amido carbon (C-11, δ 167.1), between an amido proton (10-NH, δ 6.83) and a methoxy bearing carbon (C-9, δ150.0) of B ring, and between the terminal methylene proton (H-12, δ 4.39) of the farnesyl residue and an aromatic carbon (C-4a, δ 134.4), respectively. The NOE experiment of 3 showed a correlation between the terminal methylene proton (H-12, δ 4.39) and a proton (H-6, δ 6.14) of B ring. Methanolysis of 3 by reflux with 1.5N HCl-MeOH gave compound-I (4)

which showed the molecular ion at m/z 300 (M^+) and a fragment ion at m/z 150 [M^+-C_8 H_9NO_2, base peak]. The molecular formula of 4 was found to be $C_{16}H_{16}N_2O_4$ by the HREI-MS (M^+, found m/z 300. 1110, calcd m/z 300. 1136). The ^{1}H- and ^{13}C-NMR spectra of 4 (Tables 6 and 7) indicated that 4 possessed a secondary amine (5-NH, δ 9.77) in place of a farnesyl residue in the molecule. In the ^{13}C-^{1}H long range COSY experiment of 4, the long range correlations were observed between an amino proton (5-NH, δ 9.77) and four aromatic carbons (C-5a, δ 126.8; C-6, δ 98.2; C-9a, δ 121.3; C-11a, δ 122.5), and between an amido proton (10-NH, δ 6.86) and three aromatic carbons (C-5a, δ 126.8; C-9, δ 149.7; C-11a, δ 122.5), indicating the presence of a seven-membered ring including an amine group and an amido group in the molecule of 4. According to these data, the structure of 4 was elucidated to be 4,7,9-trihydroxy-dibenzo[b,e][1,4]diazepin-11-one. Thus, the structure of BU-4664L (1) was determined as 5-farnesyl-4,7,9-trihydroxy dibenzo[b,e][1,4]diazepin-11-one. These structures are as follows:

Structures of BU-4664L, its derivatives and compound-I



	R_1	R_2
BU-4664L (1)	H	
Triacetyl BU-4664L (2)	$COCH_3$	"
Trimethyl BU-4664L (3)	CH_3	"
Compound-I (4)	CH_3	H

The Ohkuma et al. investigators performed a thorough spectral analysis of the BU-4664L molecule and the derivatives that they isolated. For all of the spectral analyses performed by Ohkuma et al. and presented in the cited reference, there is no teaching of a

correlation between the terminal methylene C-12 of the farnesyl group and the carbonyl carbon (C-11) of the A ring (paragraph 7, Point A (iv) of the Mitscher Declaration). This is the opposite of what is described in the Igarashi *et al.* reference, wherein it is stated that "[s]ubstitution of the farnesyl residue on the amido nitrogen at position 10 [of the Igarashi molecule] was unambiguously determined by the HMBC correlations from the C-12 methylene (δ_H 4.38, δ_C 47.8) to C-9a and the carbonyl carbon C-11". There is no reason to believe that the Ohkuma et al. investigators had long range COSY structural data that showed a correlation between farnesyl group C-12 and the A ring carbonyl carbon (C-11), given that other COSY long range correlation data were presented. The Ohkuma et al. investigators stated quite emphatically those, and only those, correlations that they observed. This is clearly evidenced by the wording "*the* correlations were observed *between* ...", as presented at column 14, lines 42-43 of the Okhuma et al. reference [emphasis added]. In view of this, Applicants submit that there is no reason to believe Ohkuma et al. erred in omitting the disclosure of a long range COSY correlation which necessarily would have been present if the Okhuma et al. molecule were to have the structure as hypothesized by Igarashi et al.

Applicants emphasize that the Ohkuma et al. investigators responsible for the structure determinations presented in the reference have performed structural analyses on a significant number of different secondary metabolites isolated from actinomycete species, and their work has been accepted both in the scientific community (see Feidler et al. (2005) "Marine actinomycetes as a source of novel secondary metabolites" *Antonie van Loeuwenhock* 87: 37-42, in specific regards to BU-4664L) and by the USPTO on numerous occasions. A list of peer-reviewed publications and issued patents on which the Ohkuma et al. researchers are credited with authorship and/or inventorship is provided in **Appendix I** (listing U.S. patents and literature references; literature references included herein as **Exhibits B-P**). As such, there is no reason to question the credibility of such experienced and competent researchers, especially in light of the fact that Igarashi et al.'s "comparative analyses" were done in the complete absence of any direct (i.e. actual) experimental comparison with the BU-4664L compound (discussed further below).

The NMR spectra in Ohkuma et al. and Igarashi et al. are significantly different

Fourth, the Office Action states that the rejection "is made on the compound produced by the same microorganism and has the same NMR spectrum as in the prior art (with the difference of solvent peaks)." This statement is incorrect not only because the microorganism is not the same (discussed above), but also because the NMR spectra actually differ with regard to more than the solvent peaks. In the accompanying Rule 132 Declaration, Dr. Lester Mitscher explains in detail the discrepancies between the respective NMR spectra. As Dr. Mitscher explains, solvent differences and inter-instrument variations cannot account for the differences in NMR spectra observed, particularly with regard to carbon atom 5a of the core ring structure of BU-4664L and carbon atom 11a of the core ring structure of the molecule analyzed by Igarashi et al (paragraph 7, Point A (ii) and (iii) of the Mitscher Declaration). Dr. Mitscher concludes that the ^{13}C NMR data presented by Igarashi et al. are not interchangeable with the ^{13}C NMR data presented in the Ohkuma et al. reference. He further concludes that the data do not support the re-definition of the carbon atoms of the core ring structure of BU-4664L to match that of the molecule isolated by Igarashi et al., particularly without directly testing, in a side-by-side manner, a sample of the BU-4664L compound versus the compound isolated by Igarashi et al. from TP-A0860 (see below). If the scenario is to be that as proposed by Igarashi et al., wherein BU-4664L should be redrawn to equate with the structure of the molecule isolated by Igarashi et al. from *Micromonospora* sp. strain TP-A0860, then the experimental data (*i.e.* the spectral data) presented by Okhuma et al. would not only have had to been misinterpreted by Okhuma et al., but even more so, Okhuma et al.'s spectral data must have been erroneously disclosed by being incomplete (missing COSY data) **and** inaccurate (instances of incorrect numerical values reported for four different molecules (*i.e.* the BU-4664L molecule **and** the three derivatives)). Applicants submit, and as supported by the explanation provided by Dr. Mitscher, that one of skill in the art would expect a certain degree of similarity in the spectral data between the molecule described by Ohkuma et al. and the molecule isolated by Igarashi et al. However, the data are not in complete agreement so as to thereby allow for the structure of the molecule described in Igarashi et al. (2005) to be superimposed

onto BU-4664L. There is no justification for stating, *or even implying*, that the spectral data presented in the Ohkuma et al. reference is incorrect.

Comparison of the ^{13}C NMR data for carbon 11a of the Igarashi et al. molecule (124.8) to the ^{13}C NMR data for carbon 11a of ECO-4601 disclosed in Applicants' specification (125.0) reveals a difference in values that is within the acceptable range of variation as stated by Dr. Mitscher in paragraph 7, Point A (iii) of his Declaration (a few tenths of a part per million), and excludes the possibility of clerical error in the reporting by Igarashi et al. of the ^{13}C NMR data for carbon 11a of the Igarashi et al. molecule.

Igarashi et al. did not perform any direct comparison between the compound they isolated and the Ohkuma et al. compound.

Fifth, and perhaps most significantly, Igarashi et al. do not base their conclusions as to the proposed "revised" structure of the compound reported by Ohkuma et al. on **any** direct comparison of the compound produced by strain TP-A0860 and **any** compound produced by *Micromonospora* species strain M990-6. In fact, the Igarashi et al. authors openly admit this fact at the bottom of page 351, when they state:

> "*Although the **direct comparison** with the authentic compound of BU-4664L **was not possible***, our spectral data strongly indicate that the compound produced by strain TP-A0860 is identical to BU-4664L" (emphases added).

Given that Igarashi et al. failed to present any evidence that is based upon a direct experimental comparison between the molecule which they isolated from *Micromonospora* sp. strain TP-A0860 and the molecule isolated by the Okhuma et al. investigators (from *Micromonospora* sp. strain M990-6), the conclusion made by Igarashi et al. that BU-4664L is the exact same molecule as that which Igarashi et al. isolated from TP-A0860 has no reasonable basis in fact. Dr. Mitscher reached this conclusion in his review of the matter, as described in the accompanying Rule 132 Declaration (paragraph 7, Point B (i) and (ii) of the Mitscher Declaration). Rather than both compounds (the Igarashi et al. compound from TP-A0860 and Okhuma et al.'s BU-4664L) being one and the same, Applicants submit that the data presented by Okhuma et al. is fully supportive of the structure of BU-4664L as determined by Ohkuma et al. and

presented in US Patent 5,541,181, while the compound isolated by Igarashi et al. has the structure of ECO-4601.

Unless the Igarashi et al. reference is read very carefully, the manner in which both the spectral data (Table 1) and the structural diagram (Figure 1) are presented in the reference can be very misleading. The legend that is provided for Table 1 in Igarashi et al. clearly indicates that the NMR data is that for BU-4664L. This is a mischaracterization of facts. The data presented in Table 1 of Igarashi et al. is that of the molecule isolated by Igarashi et al. It is **not** the true NMR data for BU-4664L. This is likewise for the molecule structure that is characterized as being BU-4664L by these authors in Figures 1 and 2 of the Igarashi et al. reference – the structure is that for the molecule isolated by Igarashi et al., although both Figures are labeled by Igarashi et al. as being that for BU-4664L. Applicants also note that while the title "Revision of the Structure Assigned to the Antibiotic BU-4664L from *Micromonospora*" given to the Igarashi et al. paper appears to set out a strong statement as to the actual structure of BU-4664L, the body and conclusions of the reference itself are less than unequivocal. The Igarashi et al. authors conclude "We thus ***propose*** that the structure assigned for BU-4664L in ref 1 should be revised to 5, 10-dihydro-4,6,8-trihydroxy-10-(3,7,11-trimethyl-*trans*-2, *trans*-6, 10-dodecatrienyl)-11*H*-dibenzo[*b,e*][1,4] diazapin-11-one" (emphasis added). That is, even the Igarashi et al. authors, in acknowledgment that there was no direct comparison with "authentic BU-4664L" merely *propose* a revised structure. This by no means demonstrates that the structure of BU-4664L is **necessarily** that determined by Igarashi et al. for the compound produced by TP-A0860. Because inherent anticipation requires that the element not expressly stated in the reference (here, the structure set out in claim 1) **necessarily** be present in the prior art reference, Applicants submit that the combination of Ohkuma et al. and Igarashi et al. fails to meet the accepted legal standard for anticipation of the claimed invention under §102.

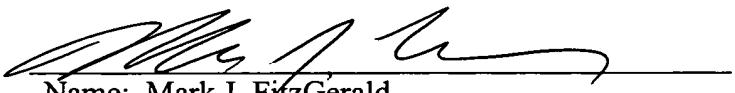
In view of the above, Applicants again emphasize that there is **no basis in fact** for the assertion made by Igarashi et al. that BU-4664L is actually the same compound as that produced by TP-A0860 and described in their publication. Under the law, the possibility that a certain characteristic or result *may* occur or be present in the prior art is

not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534 (Fed. Cir. 1993). The Court of Appeals for the Federal Circuit has stated that inherency "may not be established by *probabilities or possibilities*. The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient." *In re Robertson*, 169 F. 3d 743 (Fed. Cir. 1999), quoting *In re Oelrich*, 666 F.2d 578, 581-582 (CCPA 1981) (emphases added). In view of the shortcomings of the Igarashi et al. paper and the real possibility that strain TP-A0860 produces the compound isolated by Igarashi et al. while strain M990-6 produces the compound having the structure reported as BU-4664L, Applicants submit that not only has it not been demonstrated that the compound isolated by Igarashi et al. is ***necessarily*** the same compound as that described by Ohkuma et al., but it has not been demonstrated that the structure published by Igarashi et al. is *even* ***probably*** the "actual" structure of BU-4664L described by Ohkuma et al. That is, the law regarding anticipation requires that the un-disclosed element (i.e., the structure determined by Igarashi et al.) ***necessarily*** be present in the material described in the Ohkuma et al. reference, but the evidence presented in the cited references fails to show that the element is even ***probably*** present, let alone necessarily present. As such, Applicants submit that the Ohkuma et al. reference does not, on its own or in view of Igarashi et al., expressly or inherently anticipate the claimed invention. Reconsideration and withdrawal of the §102(b) rejection over this combination of references is respectfully requested.

In view of the above, Applicants submit that all issues raised in the Office Action have been addressed herein and that the claims are in condition for allowance. Such action is respectfully requested.

Respectfully submitted,

Date: June 23, 2006



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Appendix 1:

Other United States Patents awarded to a Patentee of US 5,541,181 (Ohkuma et al.), wherein NMR spectral data are presented and the corresponding structural determination of the isolated secondary metabolite is provided.

US 5,208,364 – issued May 4, 1993 "ANTIBIOTIC 5-LIPOXYGENASE INHIBITORS"

US 5,089,522 – issued February 18, 1992 "ANTITUMOR ANTIBIOTIC BU-3285T"

US 5,036,008 – issued July 30, 1991 "ANTITUMOR ANTIBIOTIC BU-3285T"

US 4,952,709 – issued August 28, 1990 "ANTITUMOR ANTIBIOTIC BU-3285T COMPOUNDS"

US 4,675,187 – issued June 23, 1987 "BBM-1675, A NEW ANTIBIOTIC COMPLEX"

Peer-Reviewed Scientific Publications co-authored by a Patentee of US 5,541,181 (Ohkuma et al.), wherein the NMR spectral data are presented and the structural determination of the corresponding secondary metabolite is provided. (Copies of references enclosed as Exhibits B-P)

Ohkuma, H. *et al.* (1993) 5-Hydroxyanthranilic acid derivatives as potent 5-lipoxygenase inhibitors. J. Antibiot. 46(5): 705-11. (Exhibit B)

Ueki, T. *et al.* (1993) Studies on the mode of antifungal action of pradimicin antibiotics. II. D-mannopyranoside-binding site and calcium-binding site. J Antibiot. 46(3): 455-64. (Exhibit C)

Ohkuma, H. *et al.* (1992) Sultriecin, a new antifungal and antitumor antibiotic from *Streptomyces roseiscleroticus*. Production, isolation, structure and biological activity. J Antibiot. 45(8): 1239-49. (Exhibit D)

Konishi, M. *et al.* (1991) Dynemicins, new antibiotics with the 1,5-diyn-3-ene and anthraquinone subunit. I. Production, isolation and physico-chemical properties. J. Antibiot. 44(12): 1300-5. (Exhibit E)

Hanada, M. *et al.* (1991) Maduropeptin, a complex of new macromolecular antitumor antibiotics. J. Antibiot. 44(4): 403-14. (Exhibit F)

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Pteridic Acids A and B, Novel Plant Growth Promoters with Auxin-like Activity from *Streptomyces hygroscopicus* TP-A0451

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In the course of screening for plant growth regulators from microbial secondary metabolites, we isolated pteridic acids A and B from the fermentation broth of *Streptomyces hygroscopicus* TP-A0451 as plant growth promoters with auxin-like activity[1,2]. Pteridic acids induce the formation of adventitious roots in hypocotyl of kidney beans at 1 nM as effectively as auxin (indoleacetic acid), a natural plant growth hormone. We herein describe the fermentation, isolation and structure determination of pteridic acids.

The producing organism, strain TP-A0451 was isolated from a stem of bracken, *Pteridium aquilinum*, collected in Toyama, Japan. The seed culture was incubated in a medium consisting of 1% soluble starch, 0.5% glucose, 0.3% NZ-case, 0.2% yeast extract, 0.5% tryptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization) at 30°C for 4 days on a rotary shaker (200 rpm). Three-ml aliquots of the seed culture were transferred into thirty 500-ml K-1 flasks each containing 100 ml of the production medium consisting of 0.5% glucose, 2% glycerol, 2% soluble starch, 1.5% Pharmamedia, 0.3% yeast extract, 1% Diaion HP-20 (adjusted to pH 7.0 before sterilization). Fermentation was carried out for 6 days at 30°C on a rotary shaker (200 rpm). The fermented whole broth (3 liters) was centrifuged to separate the mycelium and the supernatant. The mycelium was extracted with 80% aqueous acetone (1 liter×3). After evaporation, the resultant aqueous solution was combined with the supernatant, extracted with ethyl acetate (2 liters×3) at pH 8.0, and concentrated *in vacuo*. The residual solid was washed with acetonitrile to give 2.3 g of azalomycin B (~95% purity on HPLC) as a white powder. The aqueous layer was then extracted with ethyl acetate (1 liter×3) at pH 3.5 and the organic layer was evaporated to dryness. The residue was chromatographed on silica gel with the eluent of $CHCl_3$-MeOH (100:1~50:1). The active fractions were combined, dried and rechromatographed on ODS RP-18 with CH_3CN/0.15% KH_2PO_4 buffer (pH 3.5) (2:8~3:7). The acetone of the combined active fractions was evaporated and then extracted with ethyl acetate and concentrated *in vacuo* to give pteridic acids A (**1**, 11.2 mg) and B (**2**, 3.5 mg) as a pale yellow oil.

The physico-chemical properties of **1** and **2** are summarized in Table 1. The molecular formula of **1** and **2** was determined as $C_{21}H_{32}O_5$ based on the HRFAB-MS (**1**: $[M+H]^+$, *m/z* 365.2327, Δ −0.1 mmu; **2**: $[M+H]^+$, *m/z* 365.2326, Δ −0.2 mmu) and NMR data. The presence of $\alpha, \beta, \gamma, \delta$-unsaturated carboxylic acid was indicated by the IR (1690, 1640 cm^{-1}) and UV (λ_{max} 256 nm) spectra. The ^1H and ^{13}C NMR spectral data of **1** and **2** are shown in Table 2. Both of the ^{13}C NMR spectra of **1** and **2** displayed 21 signals which consisted of five methyls, one methylene, thirteen methines and two quaternary carbons by DEPT and HMQC experiments. The planar structure of **1** was determined by the analysis of DQF-COSY and HMBC spectra (Fig. 2). The presence of spiroketal was revealed by the ^1H-^{13}C long-range couplings from H-7, H-12, H-13, H-15 and H-19 to C-11 (96.86 ppm). In addition, the long-range couplings from H-2 and H-3 to the carbonyl carbon C-1 (171.97 ppm) confirmed the $\alpha, \beta, \gamma, \delta$-unsaturated carboxylic acid residue. The geometries of C-2/C-3 and C-4/C-5 were proved to be *E* by virtue of the coupling constants of $J_{2,3}$ (15.4 Hz) and $J_{4,5}$ (15.4 Hz).

The conformation of the six-membered ring from C-7 to C-11 was deduced from the coupling constants between H-6 and H-7 (J=10.0 Hz), H-7 and H-8 (J=2.2 Hz), H-8 and H-9 (J=4.9 Hz), and H-9 and H-10 (J=11.2 Hz). The stereochemistry at C-11 was determined by the NOEs observed between H-10 and H-12, and H-7 and H-16. Furthermore, the NOEs between H-16 and H-5, and H-16 and H-14, established the stereochemistry at C-6 and C-14, respectively, as shown in Fig. 3. **2** was determined to be the stereoisomer of **1** regarding to the spiro carbon at C-11 by the differential NOE experiments in which NOEs were observed among H-7, H-9 and H-12 but not between H-10 and H-12.

The absolute configuration of pteridic acids was determined by applying the modified Mosher's method[3]. After protecting the carboxyl residue of **1** by methylation, the hydroxyl group at C-9 was esterified with (*R*)- or (*S*)-MTPA. In the ^1H NMR spectra of MTPA esters, the positive

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Table 1. Physico-chemical properties of pteridic acids A (1) and B (2).

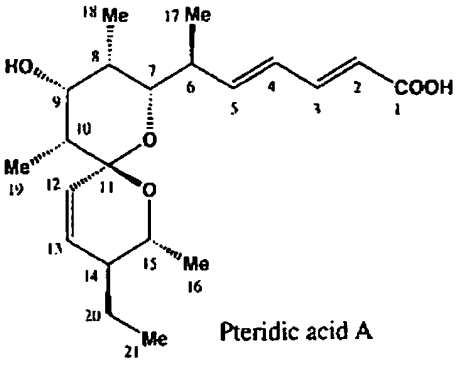
	1	2
Appearance	Pale yellow oil	Pale yellow oil
$[\alpha]_D^{24}$	-22.3 (*c* 1.0, $CHCl_3$)	-20.8 (*c* 0.68, $CHCl_3$)
HRFAB-MS		
Found:	365.2327 $[M+H]^+$	365.2326 $[M+H]^+$
Calcd:	365.2328 (for $C_{21}H_{33}O_5$)	365.2328 (for $C_{21}H_{33}O_5$)
Molecular formula	$C_{21}H_{32}O_5$	$C_{21}H_{32}O_5$
UV λ_{max}^{MeOH} nm (log ε)	256 (4.13)	256 (4.13)
IR ν_{max} (cm^{-1})	3420, 1690, 1640	3425, 1695, 1640
TLC (Rf)[a]	0.52	0.50
HPLC (Rt)[b]	7.9 min	5.2 min

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=10:1)
[b] HPLC conditions: Cosmosil AR-II (250 x 4.6 mm, i.d.), Mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (50:50), Flow rate: 1.0 ml/min, Detection: UV-254 nm.

Table 2. ^1H (400 MHz) and ^{13}C (100 MHz) NMR data for pteridic acids A and B.

Position	Pteridic acid A		Pteridic acid B	
	^{13}C	^1H	^{13}C	^1H
1	171.97		171.85	
2	118.37	5.77 (1H, d, 15.4)	118.28	5.76 (1H, d, 15.4)
3	147.47	7.25 (1H, dd, 10.0, 15.4)	147.57	7.26 (1H, dd, 10.5, 15.4)
4	126.84	6.18 (1H, dd, 9.8, 15.4)	127.44	6.24 (1H, dd, 10.5, 15.4)
5	150.11	6.25 (1H, dd, 6.8, 15.4)	149.33	6.14 (1H, dd, 7.5, 15.4)
6	38.49	2.48 (1H, ddq, 9.8, 6.8, 6.8)	38.37	2.53 (1H, ddq, 10.0, 6.8, 6.8)
7	74.48	3.75 (1H, dd, 2.2, 10.0)	75.57	3.26 (1H, dd, 2.0, 9.8)
8	36.24	2.06 (1H, ddq, 2.2, 4.6, 6.8)	36.24	2.07 (1H, ddq, 1.7, 4.9, 6.8)
9	72.48	3.85 (1H, dd, 2.2, 10.0)	74.25	3.70 (1H, dd, 4.6, 11.2)
10	40.86	1.62 (1H, quint, 6.9)	40.74	1.78 (1H, dq, 11.4, 6.8)
11	96.86		97.87	
12	127.52	5.51 (1H, dd, 1.2, 10.2)	134.00	5.92 (1H, dd, 1.9, 10.7)
13	130.25	5.96 (1H, ddd, 1.0, 5.8, 10.2)	123.43	5.89 (1H, d, 10.7)
14	40.35	1.61 (1H, dq, 11.0, 6.8)	42.26	1.86 (1H, m)
15	71.60	3.91 (1H, q, 6.8)	68.13	3.89 (1H, dq, 9.8, 6.1)
16	22.87	1.24 (3H, d, 6.8)	19.51	1.22 (3H, d, 6.1)
17	15.17	1.00 (3H, d, 6.8)	15.29	0.968 (3H, d, 6.8)
18	4.51	0.91 (3H, d, 7.0)	4.85	0.974 (3H, d, 6.8)
19	12.48	0.90 (3H, d, 6.8)	11.48	0.91 (3H, d, 6.8)
20	26.20	1.45 (2H, quint, 7.3)	23.34	1.20 (1H, m), 1.49 (1H, m)
21	11.88	0.93 (3H, t, 7.3)	9.92	0.87 (3H, t, 7.6)

Fig. 1. Structures of pteridic acids A and B.



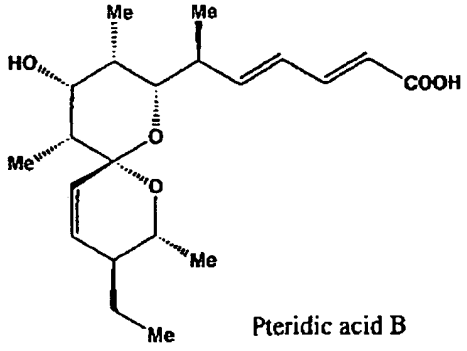
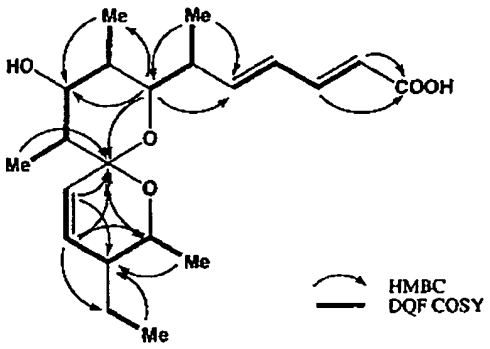


Fig. 2. DQF-COSY and HMBC correlations observed with 1.



and negative $\Delta\delta$ ($\delta_S-\delta_R$) values were well arranged on both side of the carbinyl carbon at C-9 as shown in Fig. 4. The general tendency of $\Delta\delta$ values, which were negative on the left side and positive on the right side of the MTPA plane, indicated the 9*R* configuration.

Pteridic acid is structurally related to azalomycin B (elaiophilin)[4], the major secondary metabolite of strain TP-A0451. Azalomycin B is a symmetric bislactone macrolide composed of two molecules of polyketide-derived hydroxy acids and deoxysugars. The functionalities on the carbon skelton of the hydroxy acid and pteridic acid are identical regarding to their positions and chirality except for the presence of a *cis*-olefin at C-12 and C-13 and a spiroacetal structure between C-11 carbonyl and 7- and 15-hydroxy groups in pteridic acid. Therefore, pteridic acid and azalomycin B is considered to be biosynthesized *via* a common pathway.

Experimental

Methyl Ester of Pteridic Acid A

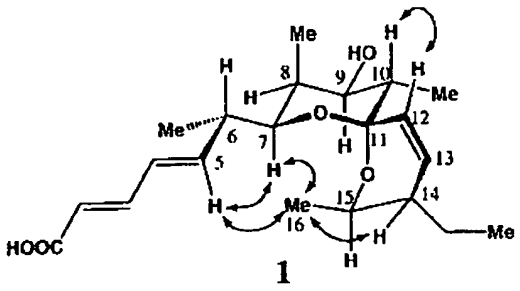
Pteridic acid A (3.6 mg, 10 μmol) was warmed with methyl iodide (25 μl, 0.4 mmol) and 1,8-diazabicyclo-[5.4.0]undec-7-ene (8 μl, 0.05 mmol) in acetonitrile (100 μl) and acetone (100 μl) at 50°C for 2 hours. The solution was poured onto ice-water and extracted with ethyl acetate. After evaporation, the residue was purified on a silica gel column (hexane-ethyl acetate=10:1~2:1) to give a methyl ester of pteridic acid A (3.2 mg, 85%).

FAB-MS: *m/z* 379 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$): 0.90 (3H, d, 7.3 Hz, H19), 0.92 (3H, d, 7.3 Hz, H18), 0.92 (3H, t, 7.4 Hz, H21), 0.99 (3H, d, 6.8 Hz, H17), 1.25 (3H, d, 6.6 Hz, H16), 1.45 (2H, quint, 7.3 Hz, H20), 1.5~1.65 (2H, m, H10 and H14), 2.06 (1H, m, H8), 2.48 (1H, ddq, 6.8, 9.8 and 9.8 Hz, H6), 3.72 (3H, s, $COOCH_3$), 3.74 (1H, dd, 2.2 and 10.0 Hz, H7), 3.84 (1H, m, H9), 3.91 (1H, q, 6.8 Hz, H15), 5.50 (1H, dd, 1.2 and 9.9 Hz, H12), 5.78 (1H, d, 15.4 Hz, H2), 5.95 (1H, dd, 5.8 and 9.9 Hz, H13), 6.16 (1H, dd, 10.0 and 15.4 Hz, H4), 6.19 (1H, dd, 6.8 and 15.4 Hz, H5), 7.18 (1H, dd, 10.0 and 15.4 Hz, H3).

MTPA Derivatization of the Methyl Ester of Pteridic Acid A

N,*N*-Dimethylaminopyridine (2.4 mg, 19 μmol), dicyclohexylcarbodiimide (5.3 mg, 25 μmol) and (*R*)- or (*S*)-MTPA acid (5 mg, 21 μmol) were added to a solution of the methyl

Fig. 3. NOEs observed with pteridic acids A (1) and B (2).



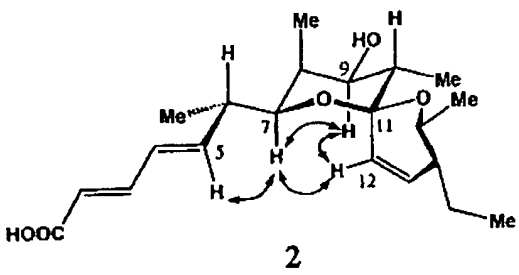
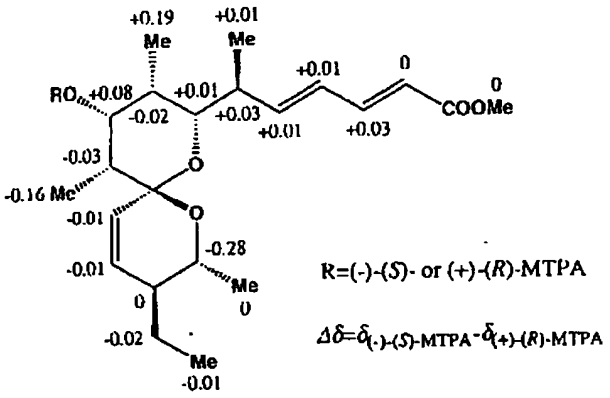


Fig. 4. Determination of the absolute configuration of pteridic acid A.



ester of pteridic acid A (1.5 mg, 4 μmol) in dry CH_2Cl_2 (100 μl). After stirring for 5 hours at room temperature, the reaction mixture was applied on a silica gel column (hexane-ethyl acetate=10 : 1~6 : 1) to give 1.4 mg (60%) of (*R*)- or 1.5 mg (65%) of (*S*)-MTPA ester.

(*R*)-MTPA ester: FAB-MS: *m/z* 595 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$): 0.719 (3H, d, 6.8 Hz, H18), 0.792 (3H, d, 6.8 Hz, H19), 0.921 (3H, t, 7.3 Hz, H21), 0.985 (3H, d, 6.8 Hz, H17), 1.245 (3H, d, 6.8 Hz, H16), 1.439 (2H, m, H20), 1.56 (1H, overlapped with H_2O signal), 1.854 (1H, m, H10), 2.304 (1H, m, H8), 2.410 (1H, m, H6), 3.550 (3H, s, CH_3O of MTPA), 3.724 (3H, s, $COOCH_3$), 3.843 (1H, dd, 2.2 and 9.8 Hz, H7), 3.938 (1H, q, 6.8 Hz, H15), 5.224 (1H, dd, 4.6 and 11.7 Hz, H9), 5.482 (1H, dd, 1.0 and 10.2 Hz, H12), 5.784 (1H, d, 15.2 Hz, H2), 5.975 (1H, dd, 5.6 and 9.5 Hz, H13), 6.145 (1H, m, H4), 6.165 (1H, m, H5), 7.144 (1H, dd, 10.0 and 15.3 Hz, H3), 7.3~7.45 (5H, m, Ph of MTPA).

(*S*)-MTPA ester: FAB-MS: *m/z* 595 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$): 0.631 (3H, d, 6.8 Hz, H19), 0.910 (3H, t, 7.3 Hz, H21), 0.910 (3H, d, 6.8 Hz, H18), 0.998 (3H, d, 6.8 Hz, H17), 1.243 (3H, d, 6.8 Hz, H16), 1.421 (2H, m, H20), 1.56 (1H, overlapped with H_2O signal), 1.827 (1H, m, H10), 2.285 (1H, m, H8), 2.438 (1H, m, H6), 3.560 (3H, s, CH_3O of MTPA), 3.662 (1H, q, 6.6 Hz, H15), 3.725 (3H, s, $COOCH_3$), 3.854 (1H, dd, 2.4 and 10.0 Hz, H7), 5.300 (1H, dd, 4.6 and 11.7 Hz, H9), 5.471 (1H, dd, 1.0 and 10.2 Hz, H12), 5.788 (1H, d, 15.2 Hz, H2), 5.961 (1H, dd, 5.4 and 9.5 Hz, H13), 6.152 (1H, m, H4), 6.172 (1H, m, H5), 7.172 (1H, dd, 10.0 and 15.4 Hz, H3), 7.33~7.45 (5H, m, Ph of MTPA).

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5-HYDROXYANTHRANILIC ACID DERIVATIVES AS POTENT 5-LIPOXYGENASE INHIBITORS

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Three new 5-lipoxygenase inhibitors, designated as BU-4601 A, B and C, were found in the fermentation broth of *Streptomyces* sp. strain No. AA2807. Their structures were identified as isodecyl, isoundecyl and isolauryl esters of 5-hydroxyanthranilic acid, respectively. Based on their structures, five related esters were synthesized and evaluated for biological activity as inhibitors of 5-lipoxygenase. Both naturally-occurring and chemically-synthesized compounds exhibited almost equal levels of 5-lipoxygenase inhibibory activities *in vitro*.

5-Lipoxygenase catalyzes the first step in the metabolic conversion of arachidonic acid into the leukotrienes which are powerful mediators of a broad range of physiological responses. Novel inhibitors of 5-lipoxygenase offer potentially useful therapeutic agents for the treatment of various diseases such as rheumatoid arthritis, ulcerative colitis, psoriasis, and asthma[1,2]. In the course of a screening program for 5-lipoxygenase inhibitors, a streptomycete (strain No. AA2807), isolated from a soil sample collected in Nagatoro, Saitama Prefecture, Japan, was found to produce three active compounds designated as BU-4601 A, B and C. Each component was isolated in a pure form by conventional column chromatography followed by preparative HPLC. The structures of BU-4601 A, B and C were determined by spectral analyses to be isodecyl, isoundecyl and isolauryl-5-hydroxyanthranilates, respectively. Based on these active lead compounds, five esters of 5-hydroxyanthranilic acid were synthesized. This paper deals with the production, isolation and structure determination of BU-4601 A, B and C, and 5-lipoxygenase inhibitory activities of three naturally-occurring and five chemically-synthesized derivatives of 5-hydroxyanthranilic acid.

Results and Discussion

Producing Organism

The producing strain AA2807 was isolated from a soil sample collected in Nagatoro, Saitama Prefecture, Japan. The morphological, cultural and physiological characteristics and cell chemistry indicated that the strain belonged to the genus *Streptomyces* (data not shown).

Production

A loopful mature slant culture of *Streptomyces* sp. strain AA2807 was inoculated into a 500 ml-Erlenmeyer flask containing 100 ml of vegetative medium consisting of soluble starch (Nichiden

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Kagaku Co.) 2.0%, glucose 0.5%, NZ-case (Humco Sheffield Chemical Co.) 0.3%, yeast extract (Oriental Yeast Co.) 0.2%, fish meal D30X (Banyu Nutrient Co.) 0.5% and $CaCO_3$ 0.3%, pH 7.0 before autoclaving. The flask was incubated at 28°C for 4 days on a rotary shaker (200 rpm) and 5 ml of the culture was transferred into a 500 ml-Erlenmeyer flask containing 100 ml of production medium (soluble starch 3.0%, beet molasses (Nihon Tensai Seito Co.) 0.5%, Protein S (Ajinomoto Co.) 2.0%, fish meal (Hokuyo Suisan Co.) 0.5% and $CaCO_3$ 0.3%, pH 7.0). The flask was incubated at 28°C for 4 days on a rotary shaker (200 rpm). Production of active materials was monitored by *in vitro* 5-lipoxygenase inhibitory activity assay.

Isolation and Purification

The fermentation broth (9 liters) was mixed well with 1-butanol (5 liters). The organic layer (4.5 liters) was separated from the aqueous layer and mycelial cake using a Sharples centrifuge (Kokusan No. 4A) and concentrated to dryness *in vacuo* to afford a crude solid (5.6 g). This solid was partitioned between ethyl acetate and water (200 ml each). The ethyl acetate layer was concentrated *in vacuo* and the residue was applied to a silica gel column (Wakogel C-200, 4.0 i.d. × 56 cm) which had been prewashed with methylene chloride. The column was developed with a linearly increasing concentration of methanol in methylene chloride from 2 to 10% in vol. The eluate was collected in 15 ml-fractions, which were assayed for 5-lipoxygenase inhibitory activity. The active fractions were collected and concentrated *in vacuo* to give a solid mixture (114 mg) of components A, B and C. The mixture (96 mg) was further subjected to preparative HPLC using a YMC-ODS, D-ODS-5 column (20 i.d. × 250 mm, YMC Co.) and the column was eluted with a linear concentration gradient of acetonitrile in water from 75 to 90%. The fractions containing a single component were combined and concentrated *in vacuo* to yield component A (2.5 mg), B (2.4 mg) or C (9.3 mg).

Physico-chemical Properties

BU-4601 A, B and C were soluble in methanol, ethanol, ethyl acetate, chlorofrom and dimethyl sulfoxide, slightly soluble in *n*-hexane, but insoluble in water. They gave positive reactions to iodine vapor, sulfuric acid and ferric chloride, but negative to ninhydrin and anthrone tests. The physico-chemical properties of three components are summarized in Table 1.

Structural Determination

BU-4601 A, B and C exhibited essentially the same UV absorption maxima at 220, 246 (sh) and

Table 1. Physico-chemical properties of BU-4601 A, B and C.

	BU-4601 A	BU-4601 B	BU-4601 C
Nature:	White amorphous powder	White amorphous powder	White amorphous powder
UV λ_{max} nm (ε)			
in MeOH:	220 (28,200), 246 (sh), 360 (5,800)	219 (24,600), 246 (sh), 360 (5,200)	219 (23,300), 246 (sh), 360 (4,800)
in 0.01 N HCl: - MeOH	211 (30,900), 233 (sh), 300 (4,400)	212 (29,200), 234 (9,100), 300 (3,800)	212 (26,000), 234 (8,000), 300 (3,600)
in 0.01 N NaOH: - MeOH	208 (34,700), 225 (24,200), 372 (4,500)	208 (33,800), 225 (24,600), 372 (4,600)	208 (33,900), 223 (21,700), 372 (4,100)
Molecular formula:	$C_{17}H_{27}NO_3$	$C_{18}H_{29}NO_3$	$C_{19}H_{31}NO_3$
EI-MS *m/z*:	293 (M^+)	307 (M^+)	321 (M^+)
HPLC (Rt)*:	4.9 minutes	6.5 minutes	9.4 minutes

* Column: A301-3-S-3 120 A ODS (4.5 i.d. × 100 mm, YMC), mobile phase: CH_3CN - H_2O = 3 : 1.

Fig. 1. IR spectrum of BU-4601 C (KBr).

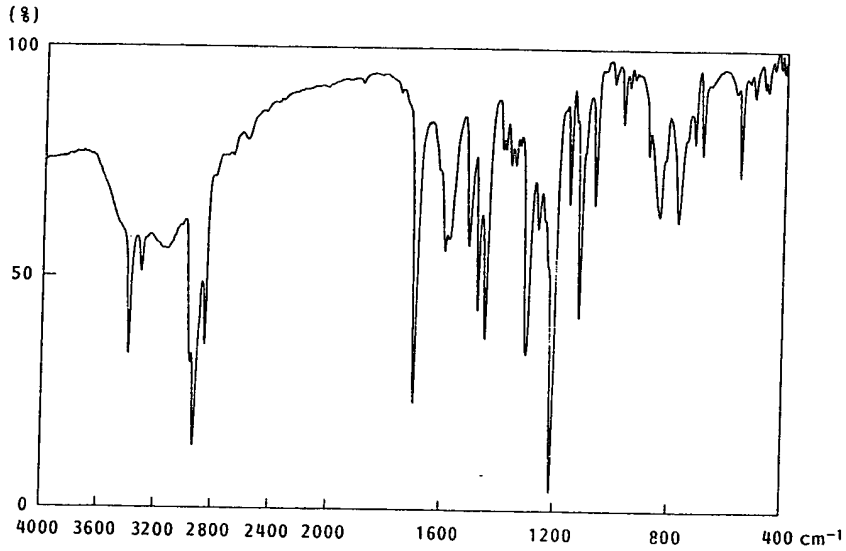


Table 2. ^{1}H NMR spectral data of BU-4601 A, B and C (400 MHz in $CDCl_3$).

Proton	BU-4601 A	BU-4601 B	BU-4601 C
1, 2-CH_3	0.86 (6H, d, J=6.5 Hz)	0.86 (6H, d, J=6.8 Hz)	0.86 (6H, d, J=6.8 Hz)
4-CH_2	1.17 (2H, m)	1.13 (2H, m)	1.15 (2H, m)
A: 5~7-CH_2	1.26	1.25	1.2
B: 5~8-CH_2	} (6H, m)	} (8H, m)	} (10H, m)
C: 5~9-CH_2	1.37	1.35	1.4
$COOCH_2CH_2CH_2$-	1.43 (2H, m)	1.43 (2H, m)	1.42 (2H, m)
3-CH	1.53 (1H, m)	1.58 (1H, m)	1.58 (1H, m)
$COOCH_2CH_2CH_2$-	1.74 (2H, m)	1.75 (2H, m)	1.74 (2H, m)
$COOCH_2CH_2CH_2$-	4.26 (2H, t, J=6.8 Hz)	4.25 (2H, t, J=6.8 Hz)	4.25 (2H, t, J=6.8 Hz)
5'-OH	4.31 (1H, brs, OH)	4.34 (1H, br, OH)	4.39 (1H, br, OH)
2'-NH_2	5.38 (2H, br, NH_2)	5.40 (2H, br, NH_2)	5.38 (2H, brs, NH_2)
3'-H	6.60 (1H, d, J=8.9 Hz)	6.60 (1H, d, J=8.5 Hz)	6.59 (1H, d, J=8.5 Hz)
4'-H	6.88 (1H, dd, J=8.9, 3.0 Hz)	6.88 (1H, dd, J=8.5, 3.0 Hz)	6.88 (1H, dd, J=8.5, 3.0 Hz)
6'-H	7.34 (1H, d, J=3.0 Hz)	7.34 (1H, d, J=3.0 Hz)	7.34 (1H, d, J=3.0 Hz)

360 nm, indicating that three components possessed the same chromophore moiety. The IR spectrum of BU-4601 C (Fig. 1) showed strong absorption bands of amino (3395 cm^{-1}), hydroxyl (3150 cm^{-1}), ester (1700, 1210 and 1020 cm^{-1}) and aromatic (1595 and 1570 cm^{-1}) functionalities. The EI-MS spectra of BU-4601 A, B and C showed molecular ions at m/z 293, 307 and 321, respectively, as well as common fragment ions at m/z 153 ($C_7H_7NO_3$) and 135 ($C_7H_5NO_2$) which were due to a benzoic acid moiety substituted with one amino and one hydroxyl groups. The ^{1}H NMR spectral data (Table 2) of these three components were very similar to each other except for methylene groups (δ 1.2~1.4). The EI-MS and ^{1}H NMR spectral data suggested that components A, B and C differed only in the number of methylene groups ($CH_2 \times 7$, $CH_2 \times 8$ and $CH_2 \times 9$, respectively). As seen in the ^{1}H NMR spectrum (Fig. 2), BU-4601 C shows three aromatic protons (3'-H, δ 6.59; 4'-H, δ 6.88 and 6'-H, δ 7.34) which are assignable to a 1,2,4-trisubstituted benzene ring from coupling constants. The spectrum also exhibits one amino (2'-NH_2, δ 5.38) and one hydroxyl (5'-OH, δ 4.39) group, suggesting a 5-hydroxyanthranilate structure based on the

Fig. 2. ^1H NMR spectrum of BU-4601 C (400 MHz, $CDCl_3$).

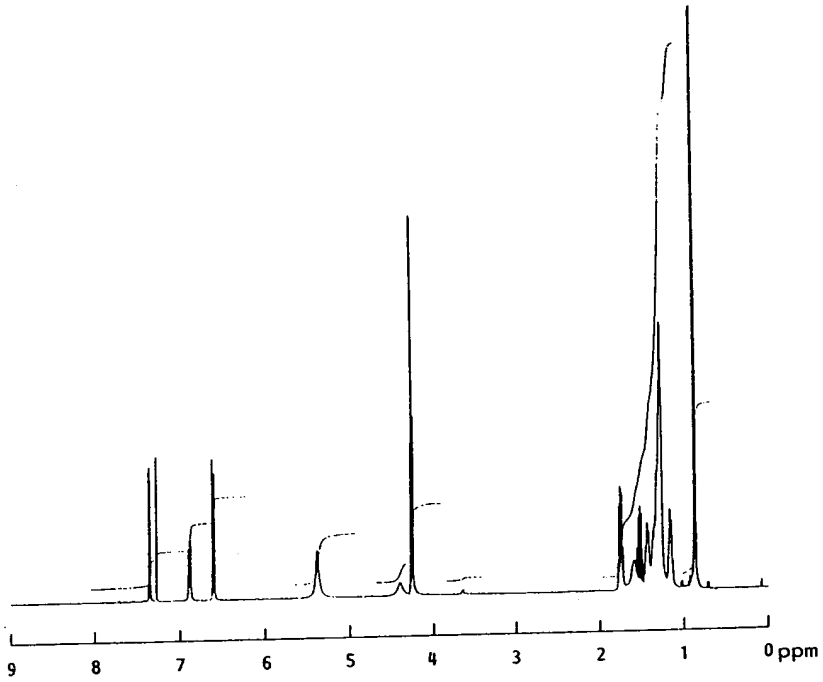
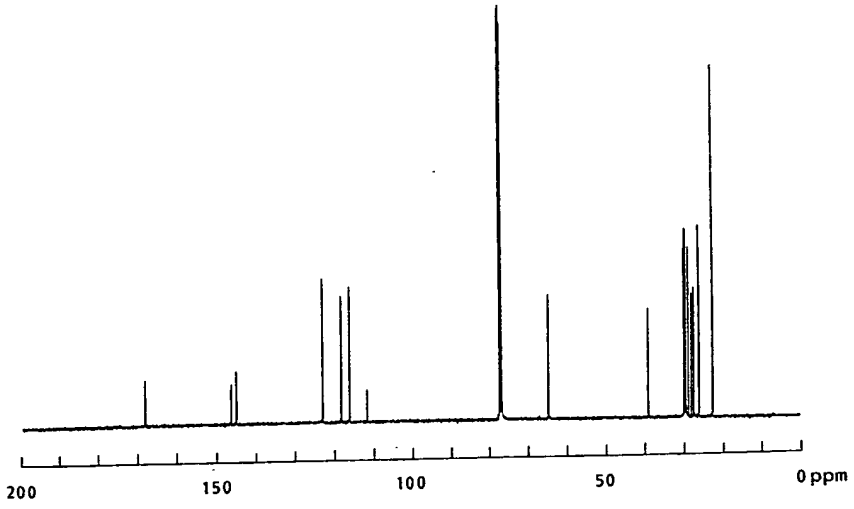


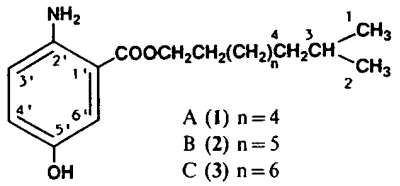
Fig. 3. ^{13}C NMR spectrum of BU-4601 C (100 MHz, $CDCl_3$).



published data[3~7]. This deduction was spectrometrically (IR, UV) confirmed by direct comparison of methyl ester of BU-4601 chromophore with the authentic sample of methyl 5-hydroxyanthranilate.

Furthermore, two methyl signals observed at δ 0.86 (1- and 2-CH_3, d, J=6.8 Hz, 6H) and a methine proton (3-H, δ 1.58) are assignable to an isopropyl group. These data together with a fragment ion (m/z 153, $M^+ - C_{12}H_{25}$) observed in the EI-MS spectrum of BU-4601 C indicate the presence of an isolauryl group in the molecule. This isolauryl moiety should be linked to 5-hydroxyanthranilic acid through an

Fig. 4. Structures of BU-4601 A, B and C.



A (1) n=4
B (2) n=5
C (3) n=6

Table 3. *In vitro* 5-LPO inhibitory activities of 5-hydroxyanthranilic acid esters.

Compound	IC_{50} (μM)
Natural	
Isodecyl ester (1)	3.4
Isoundecyl ester (2)	4.9
Isolauryl ester (3)	6.3
Synthetic	
Methyl ester (4)	8.3
n-Propyl ester (5)	7.4
n-Hexyl ester (6)	1.6
n-Nonyl ester (7)	2.0
n-Lauryl ester (8)	5.7
5-Hydroxyanthranilic acid	>100

ester bond based on the chemical shift of the terminal methylene (12-CH_2, δ4.25) and a characteristic IR absorption at 1700 cm^{-1}. The ^{13}C NMR spectrum (Fig. 3) of BU-4601 C shows two methyl (C-1 and -2, δ22.6 × 2), nine methylene (C-4 ~ C-10, δ26.1, 27.3, 28.7, 29.3, 29.5, 29.6 and 29.9; C-11, δ39.0 and C-12, δ64.7) and a methine carbon (C-3, δ27.9) signals which are assigned to an isolauryl alcohol moiety, in addition to six aromatic (C-1', δ111.6; C-2', δ146.1; C-3', δ116.1; C-4', δ122.9; C-5', δ144.9 and C-6', δ118.2) and an ester carbon (C-13, δ167.8) signals due to a 5-hydroxyanthranylate moiety. Side chains of components A and B were similarly determined to be isodecyl and isoundecyl alcohols, respectively. Thus the structures of BU-4601 A, B and C were determined as isodecyl (**1**), isoundecyl (**2**) and isolauryl 5-hydroxyanthranilates (**3**), respectively (Fig. 4).

Synthesis of Alkyl 5-Hydroxyanthranilates

The methyl ester (**4**) was obtained by treating 5-hydroxyanthranilic acid with diazomethane in dried DMF-ether mixture. The *n*-propyl derivative (**5**) was prepared from the acid by reaction with *n*-propanol in the presence of $SOCl_2$. *n*-Hexyl (**6**), *n*-nonyl (**7**) and *n*-lauryl 5-hydroxyanthranilates (**8**) were synthesized by condensation of diCbz-5-hydroxyanthranilic acid with *n*-hexyl, *n*-nonyl and *n*-lauryl alcohols, respectively, in dried DMF using dicyclohexylcarbodiimide and pyridine followed by hydrogenolysis in the presence of palladium black. Physico-chemical data of these synthetic derivatives are described in the Experimental section.

Biological Activity

Naturally-occurring and chemically-synthesized alkyl 5-hydroxyanthranilate derivatives were comparatively evaluated for their *in vitro* 5-lipoxygenase inhibitory activities. As shown in Table 3, all the derivatives show almost equal levels of activities. Precisely speaking, **6** shows the strongest activity, while **4** does the weakest activity among the compounds tested. Starting material 5-hydroxyanthranilic acid is inactive.

All the derivatives possessed very weak cytotoxic activities against human colon carcinoma (HCT-116) cells with IC_{50} > 100 μg/ml (data not shown).

Experimental

General

TLC was performed on a precoated silica gel plate (Kieselgel $60F_{254}$, E. Merck). The IR and UV spectra were recorded on a Jasco IR-810 IR spectrophotometer and a Jasco UVIDEC-610C spectrophotometer, respectively. The ^1H and ^{13}C NMR spectra were recorded on a Jeol JMN-GX400 spectrometer operated in the Fourier transform mode using TMS as the internal standard. The EI-MS

spectra were measured on a JMS-AX 505H mass spectrometer.

Methyl 5-Hydroxyanthranilate (**4**)

Diazomethane in ether was added dropwise to a solution of 5-hydroxyanthranilic acid (500 mg) in dried DMF (100 ml) under stirring. Mixing was continued for 16 hours at room temperature. The reaction mixture was evaporated *in vacuo* for removal of DMF. The residue was extracted with EtOAc (100 ml). The EtOAc extract, after washed with water, was concentrated to dryness *in vacuo*. The residue was crystallized with MeOH - water to afford pale brown needles of **4** (344 mg). TLC Rf 0.11 (CH_2Cl_2 - MeOH, 20 : 1); EI-MS *m/z* 167 (M^+, base peak); UV λ_{max}^{MeOH} (ε) 221 (10,100), 250 (sh), 361 (2,100) nm; $\lambda_{max}^{HCl-MeOH}$ 212 (12,100), 235 (sh), 301 (1,300) nm; $\lambda_{max}^{NaOH-MeOH}$ 222 (12,000), 250 (sh), 366 (2,400) nm; IR ν (KBr) cm^{-1} 3380, 3300, 2950, 1710, 1595, 1515, 1460, 1300, 1215, 1110; ^1H NMR (400 MHz, DMSO-d_6) δ 3.80 (3H, s), 6.07 (2H, br s), 6.63 (1H, d, J=8.5 Hz), 6.80 (1H, dd, J=8.5 and 3.0 Hz), 7.10 (1H, d, J=3.0 Hz), 8.66 (1H, s).

n-Propyl 5-Hydroxyanthranilate (**5**)

Thionyl chloride (1.0 ml) was added dropwise to an ice bath-cooled solution of 5-hydroxyanthrantilic acid (50 mg) in a mixture of DMF (2 ml) and *n*-propanol (3 ml) under stirring. Stirring was continued for 16 hours at room temperature and then the solution was refluxed for 2 hours. After addition of water, the reaction mixture was applied on a Diaion HP-20 column (20 ml). The column was washed with water and eluted with acetone - water (4 : 1). The appropriate fractions were pooled and evaporated *in vacuo* and purified by preparative TLC (CH_2Cl_2 - MeOH, 20 : 1) to afford a pale brown powder of **5** (13 mg). TLC Rf 0.40 (*n*-hexane - acetone, 2 : 1), 0.62 (CH_2Cl_2 - MeOH, 20 : 1); EI-MS *m/z* 195 (M^+), 153 ($M^+ - C_3H_6$), 135 ($C_7H_5NO_2$, base peak); IR ν (KBr) cm^{-1} 3370, 3300, 2980, 1700, 1595, 1510, 1450, 1300, 1210, 1120, 1070; ^1H NMR (400 MHz, $CDCl_3$) δ 1.02 (3H, t, J=7.7 Hz), 1.77 (2H, m), 4.22 (2H, t, J=6.7 Hz), 4.40 (1H, br), 5.40 (2H, br), 6.60 (1H, d, J=9.0 Hz), 6.88 (1H, dd, J=9.0 and 3.0 Hz), 7.35 (1H, d, J=3.0 Hz).

n-Hexyl 5-Hydroxyanthranilate (**6**)

Dicyclohexylcarbodiimide (40 mg) and pyridine (0.5 ml) were added to a solution of diCbz-5-hydroxyanthranilic acid (50 mg) in a mixture of *n*-hexyl alcohol (2 ml) and DMF (4 ml) under stirring. Stirring was continued for 20 hours at room temperature. The reaction mixture was partitioned between EtOAc and water (100 ml each). The organic layer was evaporated *in vacuo* and the residue was dissolved in a mixture of 5 ml of DMF and 2 ml of EtOH. Palladium black (20 mg) was added to the solution. The atmosphere was replaced by hydrogen at atmospheric pressure and stirring was continued for 16 hours at room temperature. The catalyst was removed by filtration and the filtrate was concentrated to dryness. Purification was performed by preparative TLC (*n*-hexane - acetone, 10 : 1) followed by Sephadex LH-20 chromatography with CH_2Cl_2 - MeOH (4 : 6) to afford **6** (15 mg) as a white amorphous powder. TLC Rf 0.46 (*n*-hexane - acetone, 2 : 1), 0.65 (CH_2Cl_2 - MeOH, 20 : 1); EI-MS *m/z* 237 (M^+, base peak), 153 ($M^+ - C_6H_{12}$), 135 ($C_7H_5NO_2$); IR ν (KBr) cm^{-1} 3370, 3300, 2930, 1695, 1595, 1515, 1450, 1300, 1210, 1120, 1070.

n-Nonyl 5-Hydroxyanthranilate (**7**)

Except that the *n*-hexyl alcohol was replaced by *n*-nonyl alcohol (2 ml), the same procedure as employed for synthesis of **6** was repeated yielding a white amorphous powder of **7** (14 mg). TLC Rf 0.48 (*n*-hexane - acetone, 2 : 1), 0.67 (CH_2Cl_2 - MeOH, 20 : 1); EI-MS *m/z* 279 (M^+, base peak), 153 ($M^+ - C_6H_{12}$), 135 ($C_7H_5NO_2$); IR ν (KBr) cm^{-1} 3390, 3300, 2950, 1710, 1595, 1500, 1460, 1300, 1250, 1215, 1110, 1070; ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, t, J=9.5 Hz), 1.20 ~ 1.38 (10H, m), 1.42 (2H, m), 1.72 (2H, m), 4.25 (2H, t, J=6.8 Hz), 4.45 (1H, br), 5.38 (2H, br), 6.59 (1H, d, J=9.0 Hz), 6.88 (1H, dd, J=9.0 and 3.0 Hz), 7.34 (1H, d, J=3.0 Hz).

n-Lauryl 5-Hydroxyanthranilate (**8**)

n-Lauryl alcohol (1 ml) was reacted following to the same procedure as employed for the synthesis of **6** yielding a white amorphous powder of **8** (5 mg). TLC Rf 0.53 (*n*-hexane - acetone, 3 : 1), 0.73 (CH_2Cl_2 - MeOH, 20 : 1); EI-MS *m/z* 321 (M^+, base peak), 153 ($M^+ - C_{12}H_{24}$), 135 ($C_7H_5NO_2$); ^1H NMR

(400 MHz, $CDCl_3$) δ0.87 (3H, t, J=6.6 Hz), 1.2~1.35 (16H, m), 1.41 (2H, m), 1.73 (2H, m), 4.25 (2H, t, J=6.8 Hz), 4.40 (1H, br), 5.40 (2H, br), 6.59 (1H, d, J=8.5 Hz), 6.88 (1H, dd, J=8.5 and 3.0 Hz) and 7.34 (1H, d, J=3.0 Hz).

5-Lipoxygenase Assay

The assay was done as previously reported[8]. Rat basophilic leukemia cells (RBL-1 cells ATCC CRL 1378) were grown for 5 days at 37°C in DULBECCO's modified minimal essential medium with 20% heat-inactivated calf serum. They were harvested by centrifugation and washed twice with DULBECCO's PBS containing 1 mM EDTA. The cells were resuspended in the same buffer and lyzed by sonication. The suspension was centrifuged at 13,000×g for removal of the cell debris and the supernatant was stored at −70°C. Enzyme was diluted to a desired specific activity with DULBECCO's PBS containing 1 mM EDTA, 0.9 mM ATP and 0.9 mM glutathione.

The enzyme solution (110 μl) was preincubated at 37°C for 5 minutes and a test sample in 20 μl of 10% DMSO-PBS was added. The reaction was started by addition of 5 μl of 2 mM arachidonic acid plus 25 mM calcium chloride in EtOH - water (3 : 1). After incubation for 5 minutes, the reaction was terminated by addition of 110 μl of EtOH and the mixture was centrifuged. The supernatant was analyzed for 5-hydroxyeicosatetraenoic acid by HPLC (Rainin Dynamax C18, 5 cm×0.46 cm) with a solvent of 82% MeOH - 18% 29.2 mM lithium acetate buffer, pH 6.3, at a flow rate of 1 ml/minute. Elution was spectrophotometrically monitored at 230 nm with a Gilson 115 UV detector and a Hewlett Packard 3396A integrator.

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STUDIES ON THE MODE OF ANTIFUNGAL ACTION OF PRADIMICIN ANTIBIOTICS

II. D-MANNOPYRANOSIDE-BINDING SITE AND CALCIUM-BINDING SITE

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Based on the structure-activity relationship data of BMY-28864 and related pradimicin derivatives, the calcium salt-forming ability and the D-mannopyranoside-specific visible absorption maximum shift of BMY-28864 were analysed in the ternary complex formation of BMY-28864 with D-mannopyranoside and calcium. The free C-18 carboxyl group of BMY-28864 was proved to be the sole site for binding to calcium, while no hydroxyl groups of the aglycone were involved in calcium salt formation. The stereospecific D-mannopyranoside-recognizing ability of BMY-28864 was completely abolished by removal of the C-5 disaccharide moiety, and, more particularly, of the C-5 thomosamine moiety. Close relationship of these findings with the antifungal action was also supported by the *in vitro* antifungal assay and the potassium leakage induction test.

In previous papers[1~4], the *in vitro* antifungal activities of pradimicin and benanomicin derivatives on yeasts were shown to be specifically expressed only in the presence of calcium. Using BMY-28864, a water-soluble pradimicin derivative, specific binding of the pradimicin to yeast cells was proved to depend on the ternary complex formation of BMY-28864 with mannan and calcium at a molar ratio of 2 : 4 : 1[5]. This highly stereospecific binding of BMY-28864 to the mannose unit (more generally, the specific sugar-recognizing ability of the pradimicin and benanomicin family of antibiotics) is biochemically worth studying, as it is currently unexplicable by the widely accepted concepts of receptor-ligand binding in the light of lectin and carbohydrate sciences. Lectins have been considered to recognize specific sugars based on the intrinsic properties of their peptide components, whereas the pradimicin and benanomicin family of compounds are not peptides. Under these circumstances, it is crucially important and essential to more precisely elucidate the mechanism of ternary complex formation of pradimicins with specific sugars and calcium in critical comparison with lectins. This type of knowledge is not only biochemically useful for receptology, but also clinically important from the viewpoint of selective toxicity of final pradimicin drugs in hosts, as sugars are essential cellular components of host animals to be treated with pradimicin, and assumed to exist ubiquitously at significant concentrations in a variety of forms throughout therapy.

In this paper, the structure-activity relationship of BMY-28864 and related pradimicin derivatives is analyzed for identification of the moieties of BMY-28864 responsible for binding to D-mannopyranoside and calcium and for induction of the visible absorption maximum shift. In brief, only the free C-18 carboxyl group of BMY-28864 serves to bind to calcium as salt, while the C-5 disaccharide moiety is essential for specific recognition of and binding to D-mannopyranoside.

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Materials and Methods

Fermentation and Isolation of BMY-28567 and Pradimicin Analogs

BMY-28567 (pradimicin A) and other pradimicin analogs as starting materials for chemical synthesis of pradimicin derivatives were produced by fermentation and isolated as reported in a previous paper[6].

Influences of Incubation Conditions on the Visible Absorption Peak of BMY-28864

Incubation pH: Reaction mixtures which contained 100 μM BMY-28864 with or without 1 mM calcium chloride at the indicated pH's were incubated at 30°C for 30 minutes. After incubation, the reaction mixtures were centrifuged at 14,000 rpm for 5 minutes. The supernatants were employed for determination of the visible absorption maximum of BMY-28864 by scanning visible spectrophotometry (400~600 nm) with a Beckman DU-70 UV-visible spectrophotometer.

Incubation Temperature: BMY-28864 (1.15 mM, 100 μl), 200 μl of 25 mg/ml yeast mannan (Product No. M7504, Sigma Chemical Co.), 690 μl of distilled water (pH 7.0) and 10 μl of 100 mM $CaCl_2$ were mixed and incubated for 30 minutes at the indicated temperatures for 30 minutes. Control tests were carried out without yeast mannan in the presence of 1 mM calcium chloride. After incubation, the reaction mixtures were centrifuged at 14,000 rpm for 5 minutes. The supernatants were subjected to scanning visible spectrophotometry in a wavelength range of 400 to 600 nm.

Calcium Concentration: Reaction mixtures contained 100 μM BMY-28864, 5 mg/ml yeast mannan and calcium chloride in a concentration range of 0.1 to 10 mM. Control tests contained no yeast mannan. After incubation at 30°C for 30 minutes, the reaction mixtures were centrifuged at 14,000 rpm for 5 minutes. The supernatants were employed for determination of the visible absorption maximum of BMY-28864 by scanning spectrophotometry from 400 to 600 nm.

Analytical Methods

UV-visible Spectrophotometric Analysis: BMY-28864 (1.15 mM, pH 7.0) was dissolved in distilled water or 50 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), pH 7.4. For interaction with mannan (Product No. M6882, Sigma Chemical Co.), 100 μl of 1.15 mM BMY-28864 was mixed with 200 μl of 10 mg/ml yeast mannan and 700 μl of distilled water or 50 mM MOPS, pH 7.4, in the presence and absence of 1 mM calcium chloride. After incubation at 30°C for 2 hours, the mixture was centrifuged for 5 minutes at 15,000 rpm and the supernatant was subjected to scanning UV-visible spectrophotometry in a wavelength range from 200 to 700 nm.

For interaction with a variety of simple sugars, the final sugar concentrations were all set at 200 mM, and the mixtures were centrifuged without incubation to give the supernatants which were analyzed by scanning UV-visible spectrophotometry.

Calcium Salt Formation: A pradimicin derivative (1.15 mM, 100 μl) was mixed with 100 μl of 10 mM calcium chloride and 800 μl of distilled water at pH 7.0, and incubated at 30°C for 18 hours. If precipitation was observed, the reaction mixture was centrifuged for 5 minutes at 15,000 rpm. The precipitates were washed twice with distilled water and then dissolved in 1.0 ml of DMSO. The DMSO solution was divided into two halves which were used for determination of the calcium and pradimicin contents by atomic absorption spectrometry and visible spectrophotometry at 498.4 nm, respectively[5]. The molar ratio of Ca^{2+}: Pradimicin was calculated from the contents of Ca^{2+} and pradimicin in the DMSO solution.

In Vitro Antifungal Assay: MIC on *Candida albicans* A9540 was determined by serial dilution in YNBG-PB liquid medium (Difco), pH 7.0, in the presence of 200 μM calcium chloride[5].

Potassium Leakage Induction Test: *Candida albicans* A9540 was cultivated and harvested as described in a previous paper[5]. One milliliter of the yeast cell suspension (5×10^7 cells) was mixed with 100 μl of a pradimicin solution, 10 μl of 100 mM calcium chloride and 890 μl of physiological saline. After incubation at 30°C for 2 hours, the cells and the supernatant were separated by centrifugation at 15,000 rpm and 4°C for 10 minutes. The control test was run without pradimicin derivative.

The supernatant solution was directly subjected to measurement of the potassium content by atomic absorption spectrometry. The control cells were boiled for 10 minutes at 100°C for release of the total amount of potassium into the supernatant which was subjected to atomic absorption spectrometry. In the control test, a reference potassium content of 23.2 ppm/5×10^7 cells was obtained and employed at 100% for calculation of the percent potassium leakage.

Chemical Synthesis of Pradimicin Derivatives

BMY-28864: This semi-synthetic pradimicin derivative as hydrochloride salt was prepared by the method of OKI *et al.*[7].

BMS-184497: Thionyl chloride (25 μl, 0.35 mmol) was added dropwise to a stirred solution of 100 mg BMY-28864 (0.117 mmol) in 50 ml of dry methanol. After stirring for one day at room temperature, 25 μl of thionyl chloride was added and the mixture was further stirred for one day. The solvent was removed by evaporation under reduced pressure and the residue was taken into 30 ml of ethyl ether. The insoluble matters were collected by filtration to give 102 mg (96% yield) BMS-184497 (BMY-28864 methyl ester) as hydrochloride. MP 190°C (dec.); IR ν (KBr) cm^{-1} 1725, 1610, 1290, 1050; ^1H NMR (400 MHz, DMSO-d_6) 1.38 (3H, d, J=6 Hz, 5-CH_3), 2.30 (3H, s, 3-CH_3), 2.99 (6H, s, 4'-N(CH_3)$_2$), 3.05~3.10 (3H, m, 4'-H, 2''-H and 5''-H), 3.67 (3H, s, $COOCH_3$), 3.73 (2H, dd, J=5 and 10 Hz, 17-CH_2), 3.76 (1H, dd, J=6 and 12 Hz, 5''-H), 3.91 (3H, s, OCH_3), 4.48~4.60 (4H, m, 5-H, 6-H, 1''-H and 17-H), 4.82 (1H, d, J=8 Hz, 1'-H), 6.95 (1H, d, J=3 Hz, 10-H), 7.30 (1H, s, 4-H), 7.31 (1H, d, J=3 Hz, 12-H), 8.07 (1H, s, J=7 Hz, 7-H), 8.14 (1H, d, J=7 Hz, CONH), 12.88 (1H, s, OH), 13.80 (1H, br, OH); FAB-MS m/z 885 $(M+H)^+$.

BMY-28754: Thionyl chloride (3 ml) was dropwise added to a stirred solution of 290 mg pradimicin A in 50 ml of ethanol, and was stirred for one hour at 0°C and then for a further two hours at room temperature. After concentration to dryness *in vacuo*, the residue was subjected to reversed-phase silica gel column chromatography (ODS-A60, Yamamura Chemical Lab., 2.0 × 32 cm). Products were eluted by increasing stepwise the concentration of acetonitrile in 0.15% KH_2PO_4, pH 3.5, starting with the ratio of 2:8 and ending with the ratio of 1:1. Eluate fractions containing BMY-28754 were combined and concentrated under reduced pressure for removal of the organic solvent. The aqueous concentrate was applied on a Diaion HP-20 column for desalting. After rinsing with distilled water, the product was eluted with 80% aqueous acetone (pH 3.0 with HCl). Concentration of the red eluate to dryness yielded 263 mg BMY-28754 hydrochloride. MP 218~221°C (dec.).

BMY-28946 (Desxylosyl BMY-28864) and BMY-28962 (BMY-28864 Aglycone): A solution of 50 mg BMY-28864 in a mixture of 5 ml of dioxane and 1 ml of 1 N HCl was heated for 8 hours on a steam bath and then neutralized with 1 N NaOH. After the dioxane was removed by evaporation *in vacuo*, the remaining aqueous solution was charged on a Diaion HP-20 chromatographic column (1.8 × 25 cm). The column was rinsed with water and eluted with 80% aqueous acetone, pH 3.0. Evaporation of the solvent *in vacuo* gave a deep red solid which was subjected to successive purification by reversed-phase silica gel column chromatography (ODS-A60, Yamamura Chemical Lab., 2.1 × 25 cm) with 35% CH_3CN in 0.15% phosphate buffer, pH 3.5, and Diaion HP-20 column chromatography (1.8 × 25 cm) with 80% aqueous acetone to yield 7.8 mg (17%) desxylosyl BMY-28864 (BMY-28946) and 11.5 mg (33%) of the aglycone (BMY-28962).

BMY-28946: MP >180°C (dec.); IR ν (KBr) cm^{-1} 3400, 1730, 1610, 1380, 1260, 1070; UV (0.01 N NaOH) λ_{max} nm (ε) 211 (38,100), 318 (14,200), 496 (12,500); ^1H NMR (DMSO-d_6 + D_2O) 1.23 (3H, d, J=7 Hz, 6'-CH_3), 2.29 (3H, s, 3-CH_3), 2.75 (6H, s, 4'-N(CH_3)$_2$), 3.16 (1H, m, 4'-H), 3.30 (1H, m, 2'-H), 3.74 (4H, m, 17-CH_2, 3'-H and 5'-H), 3.91 (3H, s, 11-OCH_3), 4.38~4.45 (3H, m, 5-H, 6-H and 17-H), 4.60 (1H, d, J=8 Hz, 1'-H), 6.73 (1H, d, J=3 Hz, 10-H), 6.89 (1H, s, 4-H), 7.12 (1H, d, J=3 Hz, 12-H), 7.77 (1H, s, 7-H). SI-MS m/z 740 $(M+2H)^+$.

BMY-28962: MP >200°C (dec.); IR ν (KBr) cm^{-1} 3240, 1720, 1605, 1340, 1305, 1165; UV (0.01 N NaOH) λ_{max} nm (ε) 212 (34,500), 319 (15,200), 498 (14,000); ^1H NMR (DMSO-d_6 + D_2O) 2.34 (3H, s, 3-CH_3), 3.73 (2H, m, 17-CH_2), 3.91 (3H, s, 11-OCH_3), 4.22 (1H, d, J=11.1 Hz, 5-H), 4.27 (1H, d, J=11.1 Hz, 6-H), 4.45 (1H, t, J=4.9 Hz, 17-H), 6.92 (1H, d, J=2 Hz, 10-H), 7.06 (1H, s, 4-H), 7.28 (1H, d, J=2 Hz, 12-H), 8.08 (1H, s, 7-H); SI-MS m/z 567 $(M+2H)^+$.

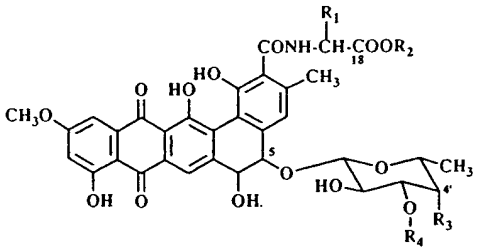
BMY-28634 (pradimicin AG-11 or B), BMY-28749 (pradimicin AG-1) and BMY-28750 (pradimicin AG-2) were prepared as described in a previous paper[8].

Results

Calcium-binding Site of BMY-28864

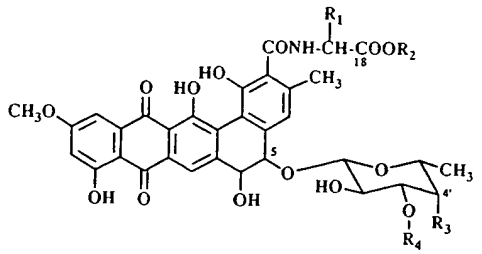
Previous results of quantitative analysis revealed that the calcium-BMY-28864-methyl α-D-

Fig. 1. Structures of BMY-28864 and related pradimicin derivatives.



Drivative	R_1	R_2	R_3	R_4
BMY-28864	CH_2OH	H	$N(CH_3)_2$	β-D-Xylose
BMS-184497	CH_2OH	CH_3	$N(CH_3)_2$	β-D-Xylose
BMY-28567	CH_3	H	$NHCH_3$	β-D-Xylose
BMY-28754	CH_3	C_2H_5	$NHCH_3$	β-D-Xylose
BMY-28946	CH_2OH	H	$N(CH_3)_2$	H
BMY-28634	CH_3	H	$NHCH_3$	H
BMY-28749	CH_3	CH_3	$NHCH_3$	H
BMY-28962	CH_2OH	H	(No sugar moiety at all)	
BMY-28750	CH_3	H	(No sugar moiety at all)	

Table 1. Analysis of the calcium-binding site.



Derivative	R_2	Ca^{2+} salt formability	Ca^{2+}/ Pradimicin
BMY-28864	H	Yes	0.50
BMS-184497	CH_3	No	0.0
BMY-28567	H	Yes	0.44
BMY-28754	C_2H_5	No	0.0
BMY-28946	H	Yes	0.48
BMY-28634	H	Yes	0.45
BMY-28749	CH_3	No	0.0
BMY-28962	H	Yes	0.57
BMY-28750	H	Yes	0.48

mannopyranoside ternary complex possesses a molar component ratio of 1 : 2 : 4[5]. For further elucidation of the mechanism of ternary complex formation, it was prerequisite to identify which moieties of the BMY-28864 molecule are involved in binding to the other two components. Without clear identification of the responsible moieties, the studies on the mode of antifungal action of pradimicin derivatives and the comparative characterization of their specific-sugar-recognizing ability would be less conclusive and meaningful particularly for future biochemical and clinical use of this interesting family of natural antibiotics and synthetic derivatives.

Using BMY-28864 and structurally-related pradimicin derivatives (see Fig. 1), the calcium-binding site of BMY-28864 was analysed first. As the terminal C-18 carboxyl group and the 4 hydroxyl groups of the aglycone of BMY-28864 were taken into account as possible calcium-binding sites, the calcium salt-forming abilities of the pradimicin derivatives were examined in the absence of mannan as the sugar component.

It is obvious from Table 1 that only the free C-18 carboxyl group of BMY-28864 is responsible for binding to calcium, as the pradimicin alkyl esters (BMS-184497, BMY-28754 and BMY-28749) form no calcium salts, whereas the other pradimicin derivatives, which all possess the free C-18 carboxyl group, yield the calcium salts at a molar Ca^{2+}/pradimicin ratio of 1 : 2. This molar ratio is identical with that observed in the ternary complex of BMY-28864 with methyl α-D-mannopyranoside and calcium[5], showing that the carboxyl group of the pradimicin derivatives stoichiometrically forms the salt with calcium. Consequently, at least as far as BMY-28864 is concerned, it is unambiguously ruled out that any one of the hydroxyl groups of the aglycone is responsible for binding to calcium. It also seems unlikely that pradimicin and benanomicin derivatives other than BMY-28864 have different calcium-binding sites other than the free C-18 carboxyl group.

D-Mannopyranoside-recognizing Site of BMY-28864

(1) UV-visible Absorption Profiles of BMY-28864 before and after Mannan-dependent Ternary Complex Formation in the Presence of Calcium.

As described in a previous paper[5], only D-mannopyranoside and D-fructose precipitated with BMY-28864 and calcium as a result of the ternary complex formation, whereas other hexoses such as D-galactose and D-glucose, and hexosamines such as 2-amino-D-mannose yielded no precipitation. It is interesting to note that the presence and configurations of the C-2 and C-4 hydroxyl groups of hexose determine precipitability of the pradimicin derivative in the form of ternary complex. As previously reported[5,9], coexistence of BMY-28864 or a pradimicin derivative with mannan and calcium induces the upward shift of the visible absorption maximum around 500 nm which is related to the characteristic red color of the benzo[*a*]naphthacenequinone ring structure.

Fig. 2 shows the UV-visible absorption spectra of BMY-28864 with calcium in the presence and absence of mannan. Spectrophotometric effect of mannan is apparent from shift of the visible absorption maximum of BMY-28864 from 499 nm to 516 nm.

Detailed effects of mannan addition on the UV-visible absorption profile of BMY-28864 are summarized as follows:

(a) Visible absorption peak of BMY-28864 shifts from 499 to 516 nm;

(b) a shoulder is generated around 560 nm;

(c) visible absorption intensity increases in a range of 300 to 440 nm;

(d) visible absorption intensity decreases in a range of 440 to 510 nm;

(e) UV absorption intensity decreases below 300 nm.

Methyl α-D-mannopyranoside produced a spectrum nearly identical to that of mannan (spectrum not shown), but differed in markedly low absorption below 300 nm.

Among these characteristics, the visible absorption maximum shift around 500 nm was selected as a general index to characterize the sugar-recognizing property of pradimicin derivatives in this paper.

(2) Reliability of the Visible Absorption Maximum Shift of BMY-28864.

Reliability of the visible absorption maximum shift of BMY-28864 as a dependable measure for

Fig. 2. Significance of mannan in the spectrophotometric profile change of BMY-28864.

a: BMY-28864 + Ca^{2+} (control), b: BMY-28864 + Ca^{2+} + mannan.

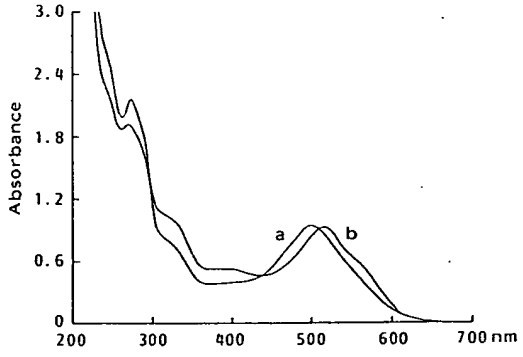


Fig. 3. Effect of the incubation pH on the visible absorption maximum of BMY-28864 in the presence and absence of calcium.

△ BMY-28864 + Ca^{2+}, ○ BMY-28864 only.

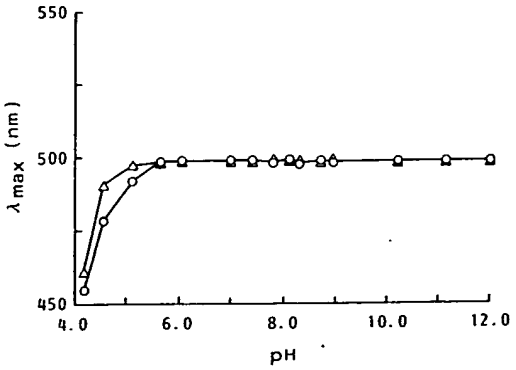


Fig. 4. Effect of the incubation temperature on the visible absorption maximum of BMY-28864 in the presence and absence of mannan.

● BMY-28864 + Ca^{2+} + mannan, ○ BMY-28864 + Ca^{2+}.

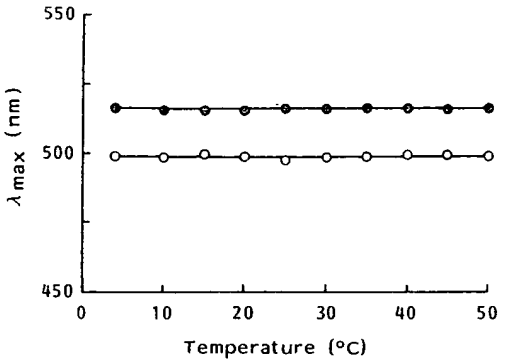
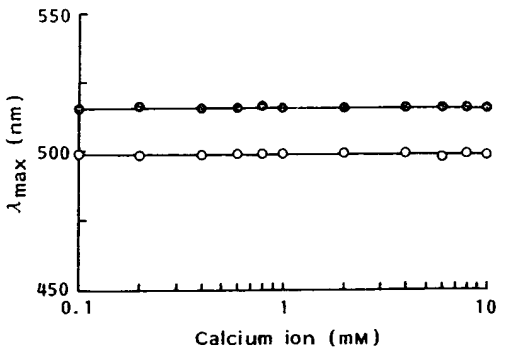


Fig. 5. Effect of the calcium concentration on the visible absorption maximum of BMY-28864 in the presence and absence of mannan.

● BMY-28864 + Ca^{2+} + mannan, ○ BMY-28864 + Ca^{2+}.



analysis of the specific-sugar-recognizing ability was examined under varied incubation conditions such as pH, temperature and calcium concentration.

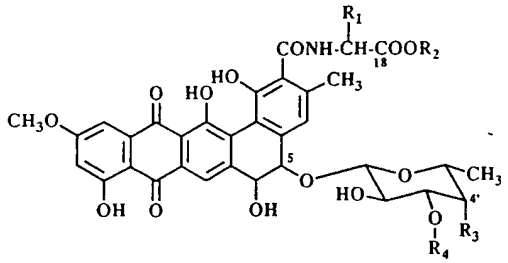
Figs. 3, 4 and 5 show that the visible absorption maximum shift of BMY-28864 is a qualitatively stable and reliable measure in wide ranges of incubation conditions such as pH (5.5~12), temperature (4~50°C) and calcium (>0.1 mM) concentration. The effect of the mannan concentration will be presented in the subsequent paper[10].

(3) Spectrophotometric Comparison of the Pradimicin Derivatives in the Mannan-dependent Visible Absorption Maximum Shift in the Presence of Calcium.

As the water-solubility greatly differs among the pradimicin derivatives employed, the sugar-dependent pradimicin precipitability is an unreliable measure for comparative analysis of the sugar-recognizing and/or sugar-binding abilities of pradimicin and benanomicin derivatives. Spectrophotometric maximum shift data, in contrast, were found to be dependable with good reproducibility, as they are not limited by water-solubility difference among the pradimicin derivatives. Results in Table 2 apparently demonstrate that the disaccharide moiety at C-5 is essential for the visible absorption maximum shift (and probably for precipitation resulting from ternary complex formation) (compare BMY-28962 and BMY-28750 with the other derivatives); and, more particularly, that the thomosamine or 4-*N*,*N*-dimethylamino-D-fucose moiety at C-5 plays a decisive role in interaction with mannan (compare BMY-28946, BMY-28634 and BMY-28749 with BMY-28864, BMY-28567 and BMY-28754, respectively). As benanomicin compounds in which the thomosamine moiety of the pradimicin analogs is replaced by D-fucose were also reported to show a visible absorption maximum shift[9], the type of the C-4′ substitute (the unsubstituted or substituted amino group versus the hydroxyl group) seems least important in the sugar-recognizing ability of the pradimicin derivatives.

It is worth mentioning that BMS-184497 (BMY-28864 methyl ester), BMY-28749 and BMY-28754 give about half (8~11 nm) the visible absorption maximum shift breadth (16 nm) as observed with BMY-28864. This clear difference in the breadth of visible absorption maximum shift served as an impetus to the subsequent study which will prove the important role of calcium in the visible absorption peak shift inducibility[10].

Table 2. Spectrophotometric analysis of the mannan-binding site.



Derivative	R_2	R_3	R_4	λ_{max} (nm) in the presence of Ca^{2+}	
				Without mannan	With mannan
BMY-28864	H	$N(CH_3)_2$	β-D-Xylose	499.3	515.6
BMS-184497	CH_3	$N(CH_3)_2$	β-D-Xylose	499.0	507.5
BMY-28567	H	$NHCH_3$	β-D-Xylose	496.0	515.4
BMY-28754	C_2H_5	$NHCH_3$	β-D-Xylose	509.4	520.5
BMY-28946	H	$N(CH_3)_2$	H	498.5	514.0
BMY-28634	H	$NHCH_3$	H	501.7	515.7
BMY-28749	CH_3	$NHCH_3$	H	499.5	507.0
BMY-28962	H	(No sugar moiety at all)		494.5	494.5
BMY-28750	H	(No sugar moiety at all)		495.7	495.7

The sugar-recognizing and/or the sugar-binding abilities of BMY-28864 are currently summarized as follows:

(a) The C-5 disaccharide (3-*O*-β-D-xylosyl-4-*N*,*N*-dimethylamino-D-fucose or xylosylthomosamine) moiety of BMY-28864 is essential for the visible absorption maximum shift, precipitability as a result of the ternary complex formation, potassium leakage inducibility and antifungal activity expression.

(b) In the two sugar constituents of the C-5 disaccharide moiety, the thomosamine is indispensable, while the D-xylose seems to be unimportantly involved in sugar recognition.

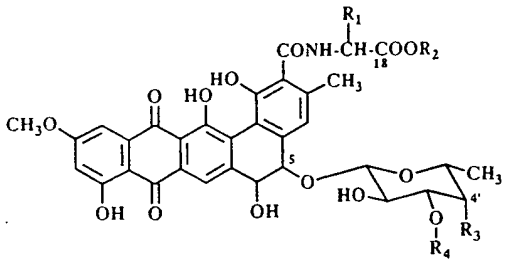
(c) As benanomicin derivatives were reported to generate the visible absorption maximum shift[9], the 4'-amino group (substituted or unsubstituted) of the thomosamine moiety is considered to play an insignificant role in sugar recognition. In other words, as far as the axial configuration is retained at C-4', a hydroxyl, amino or substituted amino group can similarly play the essential function for recognition of specific sugars.

Consistent Relationship of the Antifungal Activity and Potassium Leakage Induction Data with the Structural Requirement Data for Calcium Salt Formation and Sugar Recognition

In previous papers[2,3,5], the close relationship among pradimicin derivatives of antifungal activity and potassium leakage data with cell adsorption data was repeatedly pointed out. For example, the time course of BMY-28864-dependent potassium leakage from *Candida albicans* A9540 in Fig. 6 shows the dose dependency of potassium leakage on the BMY-28864 concentration in the presence of calcium.

Table 3 collectively compares the antifungal activity, calcium salt formability, absorption peak shift inducibility and potassium leakage inducibility of BMY-28864 and the structurally related derivatives.

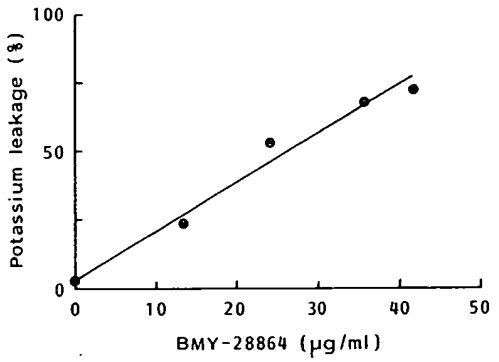
Table 3. Structure-antifungal activity relationship of pradimicin derivatives.



Derivative	R_2	R_3	R_4	MIC[a] (μg/ml)	Ca^{2+} salt formability	λ_{max} (nm) shift (with mannan and Ca^{2+})	K^{2+} leakage (%)
BMY-28864	H	$N(CH_3)_2$	β-D-Xylose	6.3	Yes	Yes (499.3→515.6)	58.5
BMS-184497	CH_3	$N(CH_3)_2$	β-D-Xylose	>100	No	Yes (499.0→507.5)	2.5
BMY-28567	H	$NHCH_3$	β-D-Xylose	3.1	Yes	Yes (496.0→515.4)	69.7
BMY-28754	C_2H_5	$NHCH_3$	β-D-Xylose	>100	No	Yes (509.4→520.4)	1.6
BMY-28946	H	$N(CH_3)_2$	H	6.3	Yes	Yes (498.5→514.0)	54.3
BMY-28634	H	$NHCH_3$	H	3.1	Yes	Yes (501.7→515.7)	29.4
BMY-28749	CH_3	$NHCH_3$	H	>100	No	Yes (499.5→507.0)	3.0
BMY-28962	H	(No sugar moiety at all)		>100	Yes	No (494.5→494.5)	0.9
BMY-28750	H	(No sugar moiety at all)		>100	Yes	No (495.7→495.7)	0.0

[a] Activity against *Candida albicans* A9540.

Fig. 6. Dependency of the potassium leakage on the BMY-28864 concentration.



In summary, the following conclusions are drawn from Table 3:

(a) Pradimicin derivatives which induce no visible absorption maximum shift in the presence of mannan (BMY-28962 and BMY-28750), even though the calcium salt-forming ability is retained, possess neither potassium leakage inducibility nor antifungal activity, probably because no binding to mannan results in nearly nil adsorptive condensation of the antibiotics on the yeast cell wall, which triggers no antifungal action on candida.

(b) Pradimicin derivatives which have no free C-18 carboxyl group (or no site for binding to calcium) (BMS-184497, BMY-28754 and BMY-28749), even though they can recognize and bind to D-mannopyranoside, are also devoid of potassium leakage inducibility and antifungal activity, presumably because, without calcium salt formability, the pradimicin derivatives cannot stay fixed on the yeast cell wall; or high concentrations of pradimicin and calcium are not generated locally on the yeast cytoplasmic membrane for expression of antifungal activity.

(c) Pradimicin derivatives which have both of the sugar-recognizing and binding ability and the calcium salt-forming ability (BMY-28864, BMY-28567, BMY-28946 and BMY-28634) express antifungal activity through some lethal actions such as potassium leakage induction as a result of ternary complex formation.

Discussion

As described in a previous paper[1], most pradimicin fermentation products are so water-insoluble that chemical modifications were inevitably attempted with success to yield virtually water-soluble pradimicin derivatives such as BMY-28864. The lipophilicity of pradimicin is largely ascribed to the pradimicin aglycone, whereas the disaccharide and amino acid moieties are intrinsically hydrophilic. Accordingly the overall lipophilicity or hydrophilicity of a pradimicin derivative results from the delicate total balance in solubility among the aglycone, the disaccharide moiety and the amino acid substituent. In practice, the pradimicin derivatives of this study greatly differ in water-solubility, which made it unacceptable to employ precipitability of a pradimicin derivative as a comparative measure for ternary complex formation. Under such circumstances, the UV-visible spectrophotometric characteristics of BMY-28864 were examined in search for a general measure to evaluate the ternary complex-forming ability. As detailed in the text, although the generation of a shoulder and the intensity change in spectrophotometric absorption are also observed before and after mannan addition, the visible absorption peak shift of *ca.* 16 nm was considered to be an appropriate replacement measure for precipitability to demonstrate comparatively the ternary complex-forming ability.

For detailed study on the mode of antifungal action of pradimicin derivatives and for further chemical derivation work, it was very interesting and urgent to identify clearly the calcium- and sugar-binding sites of BMY-28864 and other pradimicin derivatives, as the ternary complex formation is now assumed to be of absolute necessity for antifungal activity. From chemical viewpoints, some of the hydroxyl groups of the aglycone seemed to be acidic in nature and so to have a potential calcium salt-forming ability. Contrary to the authors' speculation, at least as far as the BMY-28864-related compounds are concerned, only the C-18 carboxyl group is responsible for calcium salt formation, whereas the hydroxyl groups of the aglycone probably have no effect as the alkyl esters produced no calcium salt.

As reported in a previous paper[5], it is believed that BMY-28864 and pradimicin and benanomicin derivatives, although not proteinaceous, behave like lectin, which means their sugar-binding pocket meets spacial or environmental requirements necessary for specific-sugar recognition and binding. To date, all pradimicin derivatives seem to be specific for mannose, but it cannot be explicitly ruled out that, as the antifungal activity has very often been focused on candida whose mannan is best suited for binding to pradimicin compounds, other types of pradimicin derivatives that recognize and bind to sugars other than mannose might have been left unnoticed as antifungally inactive derivatives. For now it seems reasonable to think that BMY-28864 and the antifungally active derivatives of this paper share the mannose-specific sugar binding characteristics. More particularly, the pradimicin aglycone and the thomosamine moiety of BMY-28864 serve to construct a specific mannose-binding pocket which stereospecifically recognizes and accommodates two moles of D-mannopyranoside. Lectins, on the other hand, are known to vary widely in specific-sugar recognition. Among well-characterized lectins, however, mannose-binding lectins such as concanavalin A and *Pisum sativum* lectin are assumed to be worth comparing with pradimicin derivatives, because the special requirements for the D-mannopyranoside-recognizing site would be at least partially equivalent. It is regrettable to say that no crystallographic data have yet been obtained for BMY-28864 and other pradimicin derivatives.

Structure-antifungal activity relationship study in this paper clearly demonstrates that, although there remains much to be studied, antifungally active pradimicin derivatives should have both the xylosyl-thomosamine moiety (at least the thomosamine moiety) at C-5 for recognition of and binding to mannan; and the free carboxyl group at C-18 for calcium salt formation. Once the spacial requirements for ternary complex formation of BMY-28864 are elucidated, more detailed and fruitful discussions will become possible about the substituents of the pradimicin aglycone for design of more clinically useful pradimicin derivatives.

In the meantime, BMY-28864 methyl ester or BMS-184497 gives a narrower breadth of absorption peak shift (*ca.* 8 nm) than BMY-28864 in the presence of mannan and calcium (Table 3). As the C-18 carboxyl group is protected by methyl, BMY-28864 methyl ester has no chance to form calcium salt, but its aglycone and disaccharide moiety are exactly the same as BMY-28864. Thus it is theoretically assumable that BMY-28864 methyl ester still retains the mannan-recognizing ability regardless of no calcium salt-forming ability.

This hypothesis is the starting point of the subsequent paper which will describe the reaction sequence analysis of ternary complex formation under more critical analytical conditions.

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SULTRIECIN†, A NEW ANTIFUNGAL AND ANTITUMOR ANTIBIOTIC FROM *Streptomyces roseiscleroticus*

PRODUCTION, ISOLATION, STRUCTURE AND BIOLOGICAL ACTIVITY

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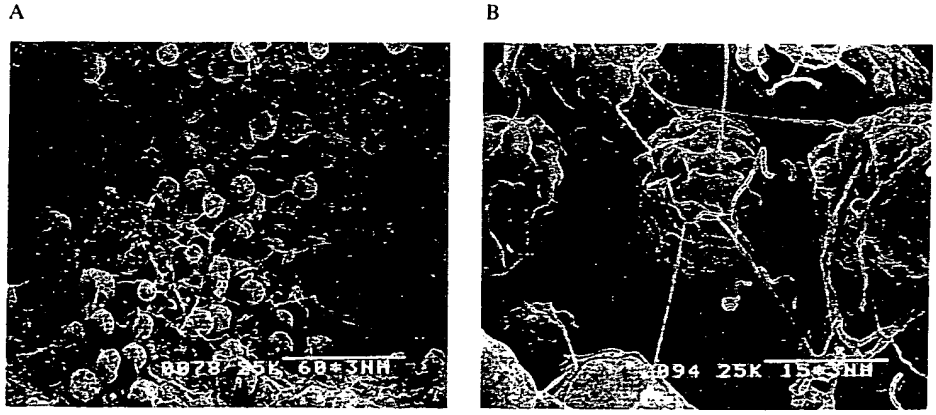
Streptomyces roseiscleroticus L827-7 (ATCC 53903) produced a novel antifungal and antitumor antibiotic, sultriecin. It exhibited *in vitro* antifungal activity and potent *in vivo* antitumor activity against P388 and L1210 leukemias, and B16 melanoma. Sultriecin is composed of several unique structural units; a conjugated triene, an α,β-unsaturated δ-lactone, and a sulfate functionality.

In our continuing search for new microbial metabolites with antitumor activity, *Streptomyces roseiscleroticus* No. L827-7, isolated from a soil sample of Gujarat State in India, was found to produce a novel antibiotic with potent *in vivo* activity against P388 and L1210 leukemias, and B16 melanoma. The active principle, sultriecin[1,2] was recovered from the fermentation broth with *n*-butanol extraction and purified by chromatography. Pentalenolactone[3,4], aburamycin[5,6] and chromomycins[7,8] were isolated as co-products from the crude extract. Structural studies by spectroscopic analysis of sultriecin and its desulfated derivative, revealed that sultriecin was 5,6-dihydro-5-hydroxy-6-(6-hydroxy-5-methyl-4-hydroxysulfonyloxyheptadec-1,7,9,11-tetraenyl)-2*H*-pyran-2-one (monosodium salt). In this paper, we describe the production, isolation, physico-chemical properties, structural determination and biological properties of sultriecin.

Fig. 1. Sclerotia of strain L827-7 grown on yeast extract-malt extract agar, 28°C for 20 days.



† Sultriecin was originally called BU-3285T.

Taxonomy of the Producing Organism

Morphology: The aerial mycelium was scantily or not formed on agar media, and when formed, it bore compact or open spiral spore chains (20~50 spores per chain). The spores were oval or oblong (0.6~0.8×1.2 μm), and had a smooth surface (Fig. 2).

The substrate mycelium was well branched and not fragmented. In the substrate mycelium, a balloon-like, semi-transparent body (3~10 μm) with a phorous hypha was numerously born and became a sclerotic globular body (10~30 μm). Scanning electron microscopy showed that the globular surface waved and had no membrane (Fig. 1, A and B). Thin section micrography indicated the globules envelop irregularly coiled and partially swollen hyphae which were cemented with an extracellular metabolite.

Cultural and physiological characteristics: The aerial mycelium was not formed on most agar media, but was seen scantily on ISP media Nos. 4 and 7. The reverse color of the substrate mycelium was deep orange to dark reddish brown. Melanin was not produced. Growth occurred between 18°C and 45°C (Tables 1 and 2).

Cell chemistry: The amino acids, sugars and phospholipids in the whole cell hydrolysate were analyzed by the methods of LECHEVALIER[9,10]. The whole cell hydrolysate was found to contain LL-2,6-diaminopimelic acid and mannose which indicated the cell wall-type I. The phospholipids detected were phosphatidylethanolamine, phosphatidylinositol and phosphatidylglycerol placing the strain in pattern P-II. The hydrolysate of globular bodies contained 2,3-diaminopropionic acid[11].

Taxonomic position: The globular bodies are identified as a sclerotium which is described in *Chainia*[11,12]. The morphology and cell chemistry

Fig. 2. Spore chain of strain L827-7 grown on tyrosine agar, 28°C for 20 days.

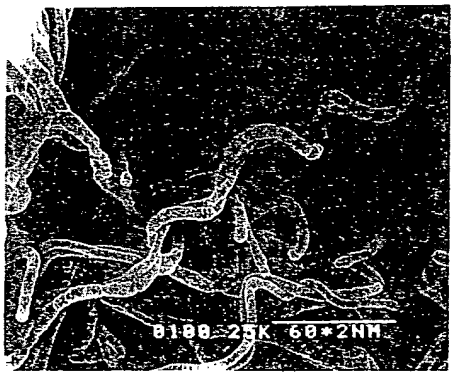


Table 1. Cultural characteristics of strain L827-7.

Medium	Growth	Aerial mycelium	Reverse color	Diffusible pigment
Sucrose-nitrate agar (Czapek-Dox agar)	Good	None	Moderate reddish-brown (43)	None
Tryptone-yeast extract broth (ISP No. 1)	Moderate, not turbid	None	Colorless	None
Yeast extract-malt extract agar (ISP No. 2)	Good	None	Deep orange (51)	Dark orange yellow (72)
Oatmeal agar (ISP No. 3)	Moderate	None	Moderate yellow (87)	Grayish yellow (90)
Inorganic salts-starch agar (ISP No. 4)	Moderate	Poor; white	Brownish orange (54)	None
Glycerol-asparagine agar (ISP No. 5)	Moderate	None	Dark reddish brown (44)	Light reddish brown (42)
Peptone-yeast extract-iron agar (ISP No. 6)	Poor	None	Colorless	None
Tyrosine agar (ISP No. 7)	Moderate	Scant; white	Dark brown (59)	Moderate brown (58)
Glucose-asparagine agar	Poor	None	Strong yellow (84)	None

Observation after incubation at 28°C for 3 weeks.
Color name in parentheses used: ISCC-NBS color name charts.

Z (14~15), E (16~17) on the basis of their coupling constants (11.3 Hz for Z, 15.1 Hz for E) assisted by the ^1H-^1H COSY experiment. Thus, the total structures of **1** and **2** have been determined as Fig. 5.

Antifungal Activity

In vitro antifungal activity was determined against various fungi by the serial 2-fold agar dilution method using SABOURAUD's dextrose agar (pH 7.0). MIC values of **1** and the reference compounds,

Table 5. Antifungal spectrum of sultriecin by an agar dilution method.

Test organism	MIC (μg/ml)		
	Sultriecin	Amphotericin B	Ketoconazole
Candida albicans IAM 4888	25	1.6	50
C. albicans A9540	25	1.6	50
Cryptococcus neoformans D49	1.6	0.8	0.4
C. neoformans IAM 4514	1.6	0.8	0.4
Aspergillus fumigatus IAM 2530	25	3.1	3.1
A. fumigatus IAM 2034	>100	3.1	6.3
Aspergillus flavus FA21436	50	1.6	0.4
Fusarium moniliforme A2284	25	3.1	6.3
Piricularia oryzae D91	50	12.5	3.1
Trichophyton mentagrophytes D155	25	6.3	1.6
T. mentagrophytes No. 4329	12.5	6.3	1.6
Blastomyces dermatidis D40	12.5	12.5	0.4
Sporothrix schenckii IFO 8158	100	>100	6.3
Petriellidium boydii IFO 8078	0.8	>100	1.6
Mucor spinosus IFO 5317	25	6.3	100

Table 6. *In vitro* cytotoxicity.

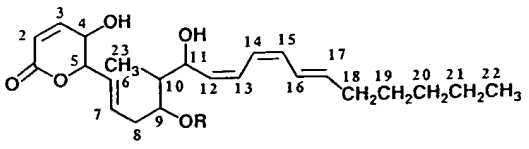
Compound	IC_{50} (μg/ml)								
	B16-F10	HCT-116	Moser	P388	P388/VCR	A549	A549/VP29	K562	K562/ADM
Sultriecin	37.7	1.92	0.85	3.80	4.40	4.14	17.0	0.67	0.37
Desulfated sultriecin	14.2	5.50	ND	ND	ND	12.7	14.7	6.7	11.10
Mitomycin C	0.50	0.80	1.20	ND	ND	ND	ND	ND	ND
Doxorubicin	0.03	0.30	ND	ND	ND	ND	ND		
VP16	0.21	5.3	ND	ND	ND	1.2	40.0	ND	ND

ND: Not tested.

Table 7. Inhibition of macromolecule biosynthesis in L1210 leukemia cells.

Compound	IC_{50} (μg/ml)		
	DNA	RNA	Protein
Sultriecin	>100	5.8	2.4
Desulfated sultriecin	>100	6.1	8.1
Mitomycin C	1.7	>100	>100

Fig. 5. Structures of sultriecin and desulfated sultriecin.



Sultriecin (**1**): R = SO_3Na
Desulfated sultriecin (**2**): R = H

Table 8. Antitumor activity of sultriecin against P388 leukemia (ip).

Compound	Dose (mg/kg/day)	Treatment schedule (ip)	MST (day)	T/C (%)	Average weight change on day 4 (g)
Sultriecin	10	QID×3	16.0	160	0.0
	3	QID×3	15.0	150	+1.5
	1	QID×3	14.0	140	+1.0
	0.3	QID×3	13.0	130	+1.0
	0.1	QID×3	12.0	120	+1.5
Mitomycin C	3	QID×3	20.0	200	−1.0
	1	QID×3	13.5	135	+0.5
	0.3	QID×3	14.0	140	+1.0
	0.1	QID×3	11.5	115	+1.8
Vehicle	—	QID×3	10.0	—	+1.3

Table 9. Antitumor activity of sultriecin against B16 melanoma (ip).

Compound	Dose (mg/kg/day)	Treatment schedule (ip)	MST (day)	T/C (%)	Average weight change on day 5 (g)
Sultriecin	10	Q3D×3	21.5	126	+0.5
	3	Q3D×3	26.5	156	0.0
	1	Q3D×3	22.5	132	+0.5
	0.3	Q3D×3	20.0	118	+0.3
	0.1	Q3D×3	18.5	109	+0.8
Mitomycin C	2	Q3D×3	34.5	203	0.0
	1	Q3D×3	23.0	135	+1.0
	0.5	Q3D×3	22.0	129	+0.3
	0.25	Q3D×3	20.0	118	+0.3
Vehicle	—	Q3D×3	17.0	—	+0.8

amphotericin B and ketoconazole against 15 different fungi are shown in Table 5. **1** exhibited moderate and broad spectrum antifungal activity, but it is not active against *Aspergillus fumigatus* IAM 2034 and *Sporothrix schenckii* IFO 8158. The desulfated derivative **2** lacks antifungal activity.

Antitumor Activity

Both **1** and **2** inhibited growth of the murine and human tumor cell lines with the IC_{50} values of 0.85~37.7 μg/ml and 5.5~14.7 μg/ml, respectively (Table 6). Among six cell lines tested, **1** exhibited the most potent cytotoxic activity against Moser (human colorectal carcinoma) and K562 (human myelogenous leukemia) cells. Interestingly, **1** and **2** showed almost the comparable cytotoxicity against the resistant (P388/VCR, A548/VP29 and K562/ADM) and their parent cell lines. The inhibitory effects of **1** and **2** on macromolecule biosynthesis were determined in cultured L1210 murine leukemia cells. As shown in Table 7, **1** and **2** primarily inhibited RNA and protein synthesis with the IC_{50} values around 2.4~8.1 μg/ml, and did not inhibit DNA synthesis at 100 μg/ml.

The *in vivo* antitumor activity was determined in tumor-bearing mice. **1** demonstrated significant prolongation of the survival time of mice inoculated with P388 (Table 8) and B16 melanoma (Table 9). The activity of **1** was comparable to mitomycin C against P388 leukemia but somewhat weaker against B16 melanoma in terms of minimum effective dose. **2** was devoid of *in vivo* antitumor activity against P388 leukemia.

Average weight change on day 4 (g)
0.0
+1.5
+1.0
+1.0
+1.5
−1.0
+0.5
+1.0
+1.8
+1.3

Average weight change on day 5 (g)
+0.5
0.0
+0.5
+0.3
+0.8
0.0
+1.0
+0.3
+0.3
+0.8

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Discussion

Sultriecin is a structurally unique antibiotic containing an α,β-unsaturated δ-lactone, a conjugated triene and a sulfate ester. It is partly related to fostriecin and its analogs[14,15] in having an α,β-unsaturated δ-lactone and a conjugated triene system, but is distinctly different from them in possessing a sulfate group. Phospholine[16], phosphazomycins[17] and phoslactomycin[18] also contain an α,β-unsaturated δ-lactone and a phosphate group and were reported to show antifungal and/or antitumor activity.

Fostriecin showed antitumor activities against P388 leukemia but no activity against solid tumors. Although sultriecin had relatively weak *in vitro* cytotoxicity, it exhibited good *in vivo* antitumor activities against P388 leukemia and B16 melanoma. It is interesting to note that the desulfated derivative exhibited *in vitro* cytotoxicity and inhibition of macromolecule biosynthesis comparable to those of sultriecin, but it did not show *in vitro* antifungal activity and *in vivo* anti-P388 effect.

Experimental

General

MP's were determined using a Yanagimoto micro-melting point apparatus and uncorrected. TLC was performed on precoated silica gel plate (Kieselgel $60F_{254}$, Merck.) The IR and UV spectra were recorded on a Jasco IR-810 IR spectrophotometer and a Jasco UVIDEC-610C spectrophotometer, respectively. The ^1H and ^{13}C NMR spectra were recorded on a Jeol JMN-GX400 spectrometer operated in the Fourier transform mode using DMSO-d_6 (δ_C 39.5, δ_H 2.50) as the internal standard. The FAB-MS spectra were measured on JMS-AX 505H mass spectrometer, and the high-resolution FAB-MS (HRFAB-MS) on a VG 70SE spectrometer. Optical rotations were determined with a Jasco Model DIP-140.

Isolation and Purification of Sultriecin (1)

The fermentation broth (58 liters, pH 7.5) was stirred with *n*-BuOH (40 liters) for one hour. The organic layer (30 liters) separated was concentrated *in vacuo* to one liter which was added dropwise into *n*-hexane (10 liters) to deposit a crude solid of the antibiotic complex (32.2 g). This was dissolved in 50% aqueous MeOH (2.8 liters) and applied on a column of Diaion HP-20 (8.0 i.d. × 20 cm) which was developed successively with 50% and 80% aqueous MeOH and 80% aqueous Me_2CO. The eluate was collected in fractions, and examined by paper disc assay using *C. albicans* A9540. The combined active fractions were concentrated *in vacuo* and the concentrate was chromatographed on a silica gel column (Wako gel C-200, 4.0 i.d. × 70 cm). The eluate was monitored on the basis of the bioassay and TLC (SiO_2, *n*-BuOH - AcOH - H_2O, 4 : 1 : 1). After elution of co-produced pentalenolactone, aburamycin and chromomycins with *n*-hexane - Me_2CO (1 : 1), sultriecin was eluted with Me_2CO. The appropriate fractions were concentrated and purified on a reversed phase silica gel column (YMC-GEL ODS-60, 4.0 i.d. × 32 cm) using acetonitrile - 1/15 M phosphate buffer, pH 7.0, (2 : 8) and then (3 : 7). The active fractions were concentrated and desalted by Diaion HP-20 to afford a semi-pure solid of sultriecin (2.2 g). The solid was dissolved in 10% aqueous MeOH (pH 9.0, 100 ml) containing EDTA (100 mg) and stirred for 3 hours. The solution was passed through a column of Diaion HP-20, and the adsorbed antibiotic was recovered by MeOH elution. The eluate was concentrated and chromatographed on a column of Sephadex LH-20 (4.0 i.d. × 40 cm) with MeOH elution. The fractions containing sultriecin were concentrated *in vacuo* to yield 753 mg of pure sultriecin as a white amorphous powder.

Enzymatic Hydrolysis of Sultriecin (1) with Sulfatase

To a solution of **1** (45 mg) in 0.1 M Tris-HCl buffer (pH 7.5, 10 ml), was added sulfatase (28 mg, S-3009 type H-5, Sigma Chemical Co.) and the mixture was incubated at 37°C for 18 hours. The reaction mixture was poured into water (40 ml) and extracted with two 50 ml portions of EtOAc. The combined extracts were evaporated to dryness *in vacuo* and the residue was purified by preparative TLC (SiO_2, CH_2Cl_2 - MeOH, 5 : 1) followed by Sephadex LH-20 chromatography using MeOH as a developing solvent to afford the desulfated sultriecin (**2**, 8 mg). **2**: White amorphous solid; IR ν_{max} (KBr) cm^{-1} 3410, 2930, 1715, 1670, 1635 and 1085; ^1H NMR (400 MHz, DMSO-d_6) δ 0.67 (3H, d, J = 6.8 Hz), 0.86 (3H, t, J = 6.5 Hz), 1.23～1.26 (5H, m), 1.36 (2H, m), 2.11 (2H, m), 2.11 and 2.20 (2H, m), 3.94 (1H, m), 4.05

(1H, m), 4.33 (1H, m), 4.81 (1H, dd, $J=7.7$ and 2.5 Hz), 5.38 (1H, m), 5.67 (1H, m), 5.71 (1H, m), 5.84 (1H, m), 5.94 (1H, d, $J=9.8$ Hz), 6.00 (1H, m), 6.15 (1H, t, $J=11.1$ Hz), 6.48 (1H, t, $J=11.1$ Hz), 6.52 (1H, dd, $J=15.2$ and 11.1 Hz) and 7.01 (1H, dd, $J=9.8$ and 5.5 Hz).

Acetylation of **1**

1 (20 mg) was stirred with acetic anhydride (0.3 ml) and pyridine (1.0 ml) for 18 hours at room temperature. The solution was diluted with EtOAc (20 ml), and washed successively with dil HCl (20 ml) and water (20 ml). The organic solution was evaporated to a residue which was purified by preparative TLC (SiO_2, CH_2Cl_2-MeOH, 10:1) followed by Sephadex LH-20 chromatography to afford di-*O*-acetylsultriecin (**3**, 12 mg). **3**: White amorphous solid, SI-MS *m/z* 577 $(M+H)^+$; IR ν_{max} (KBr) cm^{-1} 3440, 2960, 1735 and 1635; ^1H NMR (400 MHz, DMSO-d_6) δ 0.75 (3H, d, $J=7.3$ Hz), 0.84~0.87 (3H, m), 1.23~1.26 (4H, m), 1.36 (2H, m), 1.47 and 1.70 (2H, m), 2.00~2.08 (2H, m), 2.04 (6H, s×2), 2.29 and 2.65 (2H, m), 4.23 (1H, m), 5.15 (1H, m), 5.24 (1H, t, $J=10.2$ Hz), 5.28 (1H, m), 5.40 (1H, t, $J=9.6$ Hz), 5.59 (1H, m), 5.81~5.86 (2H, m), 6.09 (1H, t, $J=10.4$ Hz), 6.20 (1H, d, $J=9.8$ Hz), 6.27 (1H, t, $J=12.0$ Hz), 6.53 (1H, t, $J=15.3$ Hz), 6.58 (1H, dd, $J=12.0$ and 10.2 Hz) and 7.04 (1H, dd, $J=9.8$ and 5.5 Hz).

In Vitro Cytotoxicity and Inhibition of Macromolecule Biosynthesis

B16-F10 (murine melanoma) and Moser cells were grown in EAGLE's minimum essential medium (Nissui) supplemented with fetal calf serum (FCS, 10%) and kanamycin (60 μg/ml), A549 (human lung carcinoma), A549/VP29 (an etoposide-resistant subline of A549), and HCT-116 (human colon carcinoma) cells were grown in McCOY's 5A Medium (Gibco) supplemented with FCS (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml), and K562 and K562/ADM (an doxorubicin-resistant subline of K562), P388 (murine leukemia) and P388/VCR (a vincristine-resistant subline of P388) were in RPMI 1640 medium supplemented with FCS (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C under humidified atmosphere in a CO_2 incubator. The exponentially growing B16-F10, Moser, A549 (A549/VP29), K562 (K562/ADM), HCT-116 and P388 (P388/VCR) cells were harvested, counted and suspended in the culture media at 1.5×10^4, 2.5×10^5, 3×10^4, 3×10^4, 3×10^4 and 3×10^4 cells/ml, respectively. The test materials were planted into the wells of 96- or 24-well tissue culture plate and incubated for 72 hours. The cytotoxic activities were colorimetrically determined at 540 nm after staining viable cells with neutral red solution.

The inhibition of macromolecule biosynthesis was assessed using L1210 murine leukemia cells. The cells (5×10^5 cells/ml) were first incubated with the test materials at 37°C for 15 minutes and after the addition of the labeled precursor, [^{3}H]thymidine, [^{14}C]uridine or [^{3}H]leucine into the cultured mixture, further incubated for 60 minutes. After washing the cells with chilled 5% TCA solution, the radioactivity incorporated into the acid-insoluble fraction of the tumor cells was determined with a liquid scintillation counter.

In Vivo Antitumor Activity

B16 melanoma was intraperitoneally inoculated with 0.5 ml of 10% brei per male BDF_1 mouse, and P388 leukemia intraperitoneally inoculated with 10^6 cells per female CDF_1 mouse. The test materials were intraperitoneally administered to the tumor-bearing mice by the following treatment schedules: Once daily on days 1 to 3 (Q1D×3), once a day on days 1, 4 and 7 (Q3D×3).

Acknowledgments

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EXHIBIT E

DYNEMICINS†, NEW ANTIBIOTICS WITH THE 1,5-DIYN-3-ENE AND ANTHRAQUINONE SUBUNIT

I. PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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Dynemicin A, a novel antibiotic containing the bicyclo[7.3.1]-1,5-diyn-3-ene and 1,4,6-trihydroxyanthraquinone functionalities, was isolated from the culture broth of *Micromonospora chersina* sp. nov. M956-1. The antibiotic exhibited potent *in vitro* antibacterial and cytotoxic activity, and in *in vivo*, it cured mice from lethal *Staphylococcus aureus* infection and prolonged survival time of mice inoculated with murine tumors. Three satellite components, dynemicins L, M and N, were also isolated from the culture broth and chemically characterized.

In the first paper of this series, we reported discovery of a novel violet-colored antibiotic dynemicin A produced by *Micromonospora chersina* sp. nov. M956-1[1,2]. Dynemicin A showed potent inhibitory activity against a wide range of bacteria and various tumor cell lines. In *in vivo* test, it protected mice from lethal infection of *Staphylococcus aureus* Smith and prolonged the survival time of mice implanted with the experimental tumors. A unique structure, a hybrid of bicyclo[7.3.1]-1,5-diyn-3-ene and 1,4,6-trihydroxyanthraquinone was subsequently assigned to the antibiotic by the X-ray and spectrometric analyses[3].

In the purification of dynemicin A, three satellite components named dynemicins L, M and N were isolated as blue crystalline solid. The spectral studies disclosed that they were structurally similar to dynemicin A but the 1,5-diyn-3-ene moiety of latter was cyclized to a phenyl ring in all of the new components. Dynemicins L, M and N exhibited antibacterial and cytotoxic activity with much lower potency than that of dynemicin A.

Deoxydynemicin A was recently discovered together with dynemicin A by SHIOMI *et al.*[4] in the broth of *Micromonospora globosa* MG331-hF6. The antibiotic lacks the hydroxyl group on C-15 in our numbering system and shows strong antibacterial activity comparable to that of dynemicin A.

Herein, we present the details of fermentation, isolation and physico-chemical properties of dynemicins. The taxonomy of the producing organism and biological activity of dynemicin components are covered in the companion papers[5,6].

Fermentation

A well grown agar slant of *M. chersina*, strain No. M956-1 (ATCC 53710), was used to inoculate to a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of lactose 1%, soluble starch (Nichiden Kagaku) 3%, fish meal (Hokuyo Suisan) 1%, $CaSO_4 \cdot 2H_2O$ 0.6% and $CaCO_3$ 0.5%, the pH being adjusted to 7.0 before sterilization. The flasks were shaken at 32°C for 7 days on a rotary shaker

† Dynemicin was originally called as Bu-3420T.

(200 rpm) and a 500-ml aliquot of the culture from the flasks was inoculated into a 20-liter stir-jar fermenter containing 12 liters of the second seed medium composed of soluble starch 1.5%, glucose 0.5%, beet molasses (Nihon Tensai Seito) 1%, fish meal 1% and $CaCO_3$ 0.5% (pH 7.0). The fermentation was carried out at 28°C for 92 hours with agitation at 250 rpm and aeration of 12 liters per minute. Two liters of the second seed was transferred to a 200-liter tank fermenter containing 120 liters of production medium having the same composition as the second seed medium described above. Fermentation was carried out at 28°C for 73 hours under agitation at 250 rpm and aeration rate of 120 liters per minute. In order to follow the antibiotic production, aliquots of the fermentation broth were extracted with butanol and the extracts monitored by the paper-disc agar diffusion method using *Bacillus subtilis* PCI 219 (pH 8.0) as the indicator organism. The antibiotic production was extremely low and the maximum potency less than 1 μg/ml was obtained after 3 days.

Isolation and Purification

The fermentation broth (230 liters) was stirred for 1 hour with butanol (96 liters) and the mixture was filtered. The separated butanol layer was concentrated *in vacuo* azeotropically by occasional addition of water to 3 liters of solution which was added to ethyl acetate (18 liters) under stirring. After removal of the biologically inactive precipitate by filtration, the clear filtrate was concentrated to dryness *in vacuo* to an oily residue (124 g). It was dissolved in 70% aqueous methanol (1 liter) and the solution was charged onto a Diaion HP-20 column (1.8 liters). After washing thoroughly with 80% aqueous methanol (12 liters), the activity was eluted from the column with 80% aqueous acetone. The eluate was examined by the bioassay with *B. subtilis* PCI 219 and HPLC (column: YMC gel A301-3, 4.6 i.d. × 100 mm, solvent: MeOH - 0.15% KH_2PO_4, pH 3.5 (75 : 25)). The early eluted fractions containing dynemicins L, M and N were concentrated to yield a brown solid (3.24 g). The following most bio-active fractions were pooled and concentrated *in vacuo* to give a brown solid (15.23 g) containing dynemicin A. This solid was chromatographed on a column of Sephadex LH-20 (4.0 i.d. × 65 cm) developing with methanol. The eluate was examined by the HPLC system described above and TLC (xylene - methyl ethyl ketone - MeOH, 5 : 5 : 1). Evaporation of the faster eluted fractions gave a blue solid containing dynemicins L, M and N (351 mg). The following fractions containing dynemicin A were pooled and concentrated to yield violet solid (79 mg). Further purification of dynemicin A was effected by Sephadex LH-20 column chromatography (4.0 i.d. × 65 cm) with methanol elution. Upon examination with the HPLC, the appropriate eluates were concentrated to dryness yielding an violet solid of homogeneous dynemicin A (18 mg).

The solids containing dynemicins L, M and N were pooled (3.59 g) and applied onto a column of reversed phase silica gel (ODS-60, Yamamura Chem. Lab. 1.0 i.d. × 128 cm) prewashed with 50% aqueous methanol. Elution was carried out first with 55% aqueous methanol and then with 65% aqueous methanol. Dynemicins N, L and M were eluted with 65% aqueous methanol in the order. The HPLC-directed fractionation and evaporation of the relevant fractions resulted in the isolation of the crude solids of dynemicins N (18 mg), L (15 mg) and M (70 mg). The solid of dynemicin L was then subjected to column chromatography on silica gel (2.0 i.d. × 60 cm) using a mixture of methylene chloride - methanol (4 : 1). The fractions containing the homogeneous compound were concentrated and further chromatographed on Sephadex LH-20 (4.0 i.d. × 56 cm) with methanol elution. Evaporation of the heart-cuts of the column afforded pure dynemicin L (1.6 mg). The solid of dynemicin M was also purified by silica gel (2.0 i.d. × 35 cm, methylene chloride - methanol, 10 : 1) and Sephadex LH-20 (4.0 i.d. × 65 cm, methanol) to yield a blue solid

Table 1. Physico-chemical properties of dynemicins A, A-triacetate, L, M and N.

	Dynemicin A	Dynemicin A triacetate	Dynemicin L	Dynemicin M	Dynemicin N
Nature	Violet amorphous powder	Orange rods	Blue amorphous powder	Blue amorphous powder	Blue amorphous powder
MP (dec) (°C)	208~210	228~231	222~225	238~240	253~256
Optical rotation	$[\alpha]_D^{24}$ +270° (*c* 0.01, DMF)	$[\alpha]_D^{24}$ +1,300° (*c* 0.05, MeOH)	$[\alpha]_D^{27}$ −820° (*c* 0.01, MeOH)	$[\alpha]_D^{27}$ −2,460° (*c* 0.01, MeOH)	$[\alpha]_D^{27}$ −200° (*c* 0.01, MeOH)
UV λ_{max}^{MeOH} nm (ε)	239 (24,900), 282 (sh), 569 (10,800), 599 (10,100)	244 (40,900), 313 (7,900), 482 (8,100)	241 (48,100), 454 (2,400), 594 (18,000), 639 (17,900)	241 (41,700), 453 (1,500), 589 (17,000), 633 (17,200)	241 (53,100), 452 (4,300), 592 (24,500), 639 (25,200)
$\lambda_{max}^{0.01\,N\,HCl\text{-}MeOH}$ nm (ε)	239 (27,100), 282 (sh), 568 (11,400), 597 (10,800)	246 (37,100), 318 (7,500), 494 (7,200)	241 (47,900), 453 (1,800), 594 (17,700), 637 (17,500)	241 (41,300), 453 (1,400), 589 (16,900), 632 (17,000)	241 (52,700), 452 (3,900), 592 (24,100), 638 (25,000)
$\lambda_{max}^{0.01\,N\,NaOH\text{-}MeOH}$ nm (ε)	246 (27,900), 276 (7,400), 598 (13,300), 642 (13,800)	215 (37,600), 245 (42,700), 272 (sh), 592 (15,900), 641 (15,600)	242 (47,000), 450 (sh), 612 (18,900), 654 (20,500)	243 (44,200), 450 (sh), 607 (19,200), 655 (22,400)	243 (56,900), 450 (sh), 605 (18,300), 651 (28,300)
Molecular formula	$C_{30}H_{19}NO_9$	$C_{36}H_{25}NO_{12}$	$C_{30}H_{22}NO_9Cl$	$C_{29}H_{23}NO_9$	$C_{30}H_{23}NO_{10}$
Microanalysis		$C_{36}H_{25}NO_{12}\cdot H_2O$	$C_{30}H_{22}NO_9Cl\cdot 2H_2O$		
		Calcd: Found:	Calcd: Found:		
C:		63.43 63.20	58.68 58.86		
H:		3.99 3.75	4.27 4.00		
N:		2.06 2.16	2.28 1.93		
Cl:			5.78 5.56		
HRFAB-MS	Calcd: 538.1138				
$((M+H)^+)$	Found: 538.1132				
SI-MS (*m/z*)	538 $(M+H)^+$	664 $(M+H)^+$	576 $(M+H)^-$	532 $(M+3H)^+$	558 $(M+H)^-$
TLC, SiO_2 (Rf)[a]	0.40	0.33	0.15	0.63	0.08
HPLC (Rt: minutes)[b]	10.9		9.4	8.3	7.2

[a] Merck: Xylene - methyl ethyl ketone - MeOH (5 : 5 : 1).

[b] Column: A 301-3S-3-120A ODS (4.6 × 100 mm, YMC); mobile phase: MeOH - 0.15% KH_2PO_4, pH 3.5 (75 : 25); detection: UV 254 nm; flow rate: 1 ml/minute.

Dynemicin A
Triacetyldyne

sample of pur
TLC (Kieselg
was scraped
Evaporation

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A was purifie
homogeneous
of triacetyldyn

Dynemic
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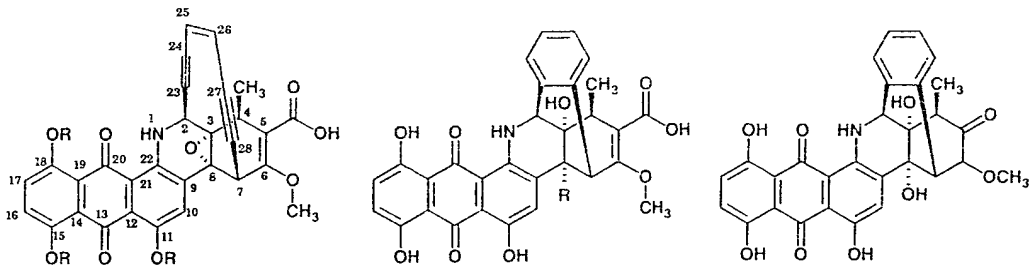
The structures
of triacetyldyn

Our ferm

Fig. 1. The structures of dynemicins A, L, M and N and triacetyldynemicin A.



Dynemicin A R=H
Triacetyldynemicin A R=$COCH_3$

Dynemicin L R=Cl
Dynemicin N R=OH

Dynemicin M

sample of pure dynemicin M (16 mg). Purification of the dynemicin N sample was effected by preparative TLC (Kieselgel $60F_{254}$, xylene-methyl ethyl ketone-methanol, 5:5:2). The appropriate band (Rf 0.18) was scraped and dynemicin N was eluted out with a mixture of methylene chloride-methanol (4:1). Evaporation of the eluate yielded a homogeneous sample of dynemicin N (2.3 mg).

Triacetyldynemicin A

Dynemicin A (15 mg) was dissolved in acetic anhydride (1.5 ml) and pyridine (2 ml) and the mixture was allowed to stand at room temperature for 18 hours. The solution was diluted with ethyl acetate (10 ml) and washed with water. The organic layer was evaporated to dryness *in vacuo*. The crude triacetyldynemicin A was purified by preparative TLC (SiO_2 plate, xylene-methyl ethyl ketone-methanol, 5:5:1) and the homogeneous orange solid (8 mg) obtained was crystallized from aqueous acetonitrile to give orange rods of triacetyldynemicin A (7 mg).

Physico-chemical Properties

Dynemicin A was obtained as a violet amorphous solid, while dynemicins L, M and N as blue amorphous solids. Dynemicins A, L, M and N are soluble in dimethyl sulfoxide, *N*,*N*-dimethylformamide and dioxane, silightly soluble in ethyl acetate, methanol and ethanol but insoluble in water and *n*-hexane. When treated with acetic anhydride in pyridine, dynemicin A yielded the triacetyl derivative with increased solubility. The molecular formula of triacetyldynemicin A was established as $C_{36}H_{25}NO_{12}$ based on HRFAB-MS, microanalysis and ^{13}C NMR data and therefore, that of dynemicin A was as $C_{30}H_{19}NO_9$ by comparative spectral analysis of both compounds. The molecular formulae of dynemicins L, M and N were determined as $C_{30}H_{22}NO_9Cl$, $C_{29}H_{23}NO_9$ and $C_{30}H_{23}NO_{10}$, respectively, by the microanalysis FAB-MS, and ^1H and ^{13}C NMR spectra. The physico-chemical data of dynemicin A and its acetate and dynemicins L, M and N are summarized in Table 1. The UV spectrum of dynemicin A exhibited absorption maxima at 239, 287, 569 and 599 nm in methanol and acidic methanol and 246, 276, 589 and 642 nm in alkaline methanol, while those of dynemicins L, M and N were exhibited the maxima at around 241, 454, 591 and 637 nm in methanol and acidic medium and around 243, 613 and 661 nm in alkaline medium. The structures of dynemicins A, L, M and N have been determined by the X-ray chrystallographic analysis of triacetyldynemicin A and spectral data comparison of the four antibiotics[2] (Fig. 1).

Discussion

Our fermentation screening directed to potent *in vivo* P388 activity resulted in discovery of another

diynene-containing antibiotic, dynemicin A. Two bicyclo[7.3.1]-1,5-diyn-3-ene antibiotics, esperamicins[7] and calicheamicins[8] were recently reported and attracted considerable interest due to their exceptionally potent antitumor activity[9], novel structures and unprecedented action mechanism[10]. Neocarzinostatin chromophore, the active principle of macromolecular antibiotic neocarzinostatin[11], is now recognized to be a member of this family in its chemical structure and mode of action.

Dynemicin A appears to be a new addition to the family of diynene antibiotic, but it is distinctly different from the preceding ones in its structure and activity profile. Dynemicin A has a number of unique structural features; unlike the preceding 1,5-diyn-3-ene containing antibiotics, the bicyclo-1,5-diyn-3-ene moiety of dynemicin A is assembled with 1,4,6-trihydroxyanthraquinone to form a tetracyclo nucleus. The absence of sugar is another structural uniqueness of this antibiotic. The trigger for activation of dynemicin A is believed to be reduction of the anthraquinone to the corresponding hydroquinone, which rearranges to a quinone methide with concomitant opening of the epoxide. Subsequent saturation of the C-8 to sp^3 carbon induces the Bergman cyclization to produce a phenyldiyl radical which cleaves DNA as in the cases of the esperamicin group antibiotics[12]. Isolation of the aromatized compound dynemicin H[12] seems to evidence the reaction. Involvement of ionic species in triggering this reaction is illustrated by the formation of dynemicins L and N upon mild acid treatment of dynemicin A[3]. The anthraquinone chromophore might also play an active role in the interaction of dynemicin A with DNA, which will be the subject of future papers.

The esperamicin/calicheamicin antibiotics are known to be strongly toxic to the mammalian. It is of particular interest that dynemicin A exhibited rather weak acute toxicity in mice[6] compared to its strong cytotoxic effect comparable to that of esperamicins and calicheamicins. It should also be emphasized that dynemicin A cured mice from lethal infection caused by *Staphylococcus aureus* Smith[1].

Acknowledgments

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EXHIBIT F

MADUROPEPTIN†, A COMPLEX OF NEW MACROMOLECULAR ANTITUMOR ANTIBIOTICS

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Maduropeptin, a complex of new macromolecular antitumor antibiotics, is a metabolite of *Actinomadura madurae* H710-49. The active components maduropeptins A_1, A_2 and B are acidic chromopeptides with MW of around 22,500 and composed of 14 types of amino acids and an unstable chromophore. The antibiotics are active *in vitro* against Gram-positive bacteria and highly cytotoxic to tumor cells. They produced significant prolongation of survival time of mice implanted with P388 leukemia and B16 melanoma.

In the course of screening for novel metabolites active against murine P388 leukemia in mice, we found a complex of new macromolecular antibiotics designated maduropeptin. The producing culture H710-49, isolated from a soil sample collected in Germany, was identified as *Actinomadura madurae* (ATCC 39144). Maduropeptin is an acidic macromolecular substance from its extraction behavior; the active principle was recovered from the fermentation broth by use of a basic ion exchange resin. HPLC analysis indicated that the crude solid contained at least four active components (maduropeptins A_1, A_2, B and D) and an inactive component (maduropeptin C) having similar physico-chemical properties. Upon UV irradiation in a cold room, components B and D were decomposed yielding a complex of components A_1, A_2 and C which were isolated as single entities by chromatography. The bioactive maduropeptin components showed MW's around 22,500 and characteristic UV absorption maxima at 210, 286 and 308 nm. They exhibited potent inhibitory activity against Gram-positive bacteria and tumor cells and strong *in vivo* antitumor effect against P388 leukemia and B16 melanoma implanted in mice. In this paper, we report the producing organism, production, isolation, chemical properties and biological activities of maduropeptin.

Producing Organism

An actinomycete strain, No. H710-49 (ATCC 39144), was isolated from a soil sample collected in Germany. Strain H710-49 forms both substrate and aerial mycelia. The substrate mycelium is long, branched and not fragmented into short filaments. Short spore-chains are born on the tip or monopodial branch of the aerial mycelium. The spore-chains contain 2~15 spores (mostly 4~8 spores), per chain, and are straight, hooked or spiral in shape. The spores are oval to elliptical ($0.5\sim0.6\times0.7\sim1.2\,\mu$m) with

† Maduropeptin was originally called as BBM-1644.

Fig. 1. Spore-chains of strain H710-49 (14 day-culture on inorganic salts - starch agar; 600 ×).

Fig. 2. Transmission electron micrograph of warty spores of strain H710-49 (14 day-culture on inorganic salts - starch agar. Bar: 1 μm).

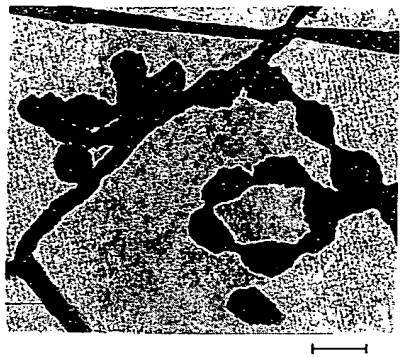


Table 1. Cultural characteristics[a] of strain H710-49.

Tryptone - yeast extract broth (ISP No. 1)	G:	Poor to moderate; floccose, sedimented and not pigmented
Sucrose - nitrate agar (CZAPEK's agar)	G:	Scant
	R:	Colorless to pale orange yellow (733)[b]
	A:	Scant; white (263)
	D:	None
Glucose - asparagine agar	G:	Poor
	R:	Yellowish white (92) to deep orange yellow (69)
	A:	Very scant; white (263)
	D:	None
Glycerol - asparagine agar (ISP No. 5)	G:	Poor to moderate
	R:	Pale yellow (89) to dark orange yellow (72)
	A:	Poor; white (263) to pale yellowish pink (31)
	D:	Brilliant yellow (83)
Inorganic salts - starch agar (ISP No. 4)	G:	Poor to moderate
	R:	Colorless to deep yellow (85)
	A:	Poor; pinkish white (9) to pale yellowish pink (31)
	D:	None
Tyrosine agar (ISP No. 7)	G:	Moderate
	R:	Brownish orange (54) to moderate reddish brown (43)
	A:	Poor; white (263) to pale yellow (89)
	D:	Strong yellow (84)
Yeast extract - malt extract agar (ISP No. 2)	G:	Moderate
	R:	Dark yellow (88) to dark brown (59)
	A:	Scant; white (263)
	D:	Light olive brown (94)
Oatmeal agar (ISP No. 3)	G:	Poor
	R:	Colorless
	A:	Poor; white (263) to pinkish white (9)
	D:	None
Peptone - yeast extract - iron agar (ISP No. 6)	G:	Poor
	R:	Grayish yellow (90) to dark grayish brown (62)
	A:	Poor; white (263)
	D:	None to moderate yellowish brown (77)

Abbreviations: G, Growth; R, reverse color; A, aerial mycelium; D, diffusible pigment.

[a] Observed after incubation at 28°C for 3 weeks.

[b] Color and number in parenthesis: ISCC-NBS color-name charts.

Table 2. Physiological characteristics of strain H710-49.

Production of:		D-Glucose	+
Amylase	+	Glycerol	+
Gelatinase	+	Inositol	−
Nitrate reductase	+	Lactose	−
Melanin	−	D-Mannitol	+
Growth in/at:		D-Mannose	−
Lysozyme, 0.001%	+	D-Melezitose	−
NaCl, 1%~7%	+	Melibiose	−
10%	−	Raffinose	−
20°C~37°C	+	L-Rhamnose	+
10°C and 41°C	−	D-Ribose	+
Utilization of*:		Salicin	−
D-Arabinose	−	Soluble starch	+
L-Arabinose	+	D-Sorbitol	−
Cellobiose	+	L-Sorbose	−
Cellulose	−	Sucrose	−
Dulcitol	−	Trehalose	+
D-Fructose	+	D-Xylose	+
D-Galactose	−		

* Basal medium: PRIDHAM-GOTTLIEB inorganic salts medium.

a round or pointed end and a warty surface. Mature spores are often separated by empty hyphae. (Figs. 1 and 2). Terminal swellings of the hyphae are occasionally observed on the substrate mycelium in CZAPEK's agar and BENNETT's agar. Motile spores, sporangia or sclerotic granules were not seen in any media examined. The cultural and physiological characteristics of strain H710-49 are shown in Tables 1 and 2, respectively.

Purified cell-wall of strain H710-49 contains *meso*-diaminopimelic acid but lacks glycine. The whole cell hydrolysate shows the presence of madurose (3-*O*-methyl-D-galactose), glucose, ribose and a small amount of mannose. The cell-wall composition and whole cell sugar components of strain H710-49 indicate that the strain belongs to cell-wall type III_B. The phospholipids contain phosphatidylinositol and phosphatidylglycerol, but not nitrogenous phospholipids, hence belong to type P-I. The menaquinone contains 68% of MK-9 (H_6) and 20% of MK-9 (H_8). The above-described characteristics of strain H710-49 resemble those of members of the genus *Actinomadura* LECHEVALIER et LECHEVALIER 1970[1]. According to the taxonomic description of known *Actinomadura* species[2~5], strain H710-49 is similar to *Actinomadura cremea*, *Actinomadura madurae* and *Actinomadura verrucosospora*. As shown in Table 3, further comparisons of the strain to the three species revealed that the strain is partially different from them, but is most similar to *A. madurae*. The strain is differentiated from *A. madurae* only in the absence of melanin formation and the lack of sucrose utilization.

Antibiotic Production

An agar slant with well-established growth of *A. madurae* H710-49 (ATCC 39144) was used to inoculate seed medium (100 ml in a 500-ml Erlenmeyer flask) containing mannitol 1%, peptone 2% and yeast extract 1%; the pH was adjusted to 7.2 before autoclaving. The seed culture was incubated at 32°C for 72 hours on a rotary shaker (250 rpm). Five ml of the mature culture was transferred to the second seed medium (100 ml) with the same composition as the first seed medium, and the seed was cultivated under the same conditions. Five ml of the inoculum growth thus prepared was employed to start fermentation in 500-ml Erlenmeyer flasks containing 100 ml of fermentation medium composed of mannitol 2.5%,

Table 3. Characteristics of strain H710-49 and three related species of *Actinomadura*.

	Strain H710-49	*A. cremea*	*A. madurae*	*A. verrucosospora*
Morphology:				
Spore chains	Hook or spiral	Hook or spiral	Hook or spiral	Hook or spiral
(No. of spores per chain)	(2~15)	(3~8)	(3~12)	(5~12)
Spore surface	Warty	Warty	Warty	Warty
Cultural property[a]:				
ISP media				
Aerial mycelium	w, p, y	w, y, p	w, p	w, p, bl
Substrate mycelium	d, y, o, b	d, b	d, p. b, w, gy	y,.o, p
Soluble pigment	—, y, b	—	—	—
Physiological characteristics:				
Melanin production	−	+	+	−
Nitrate reduction	+	+	+	−
Starch hydrolysis	+	−	+	+
Growth at 7% NaCl (w/v)	+	−	v[b]	−
Utilization of:				
Cellobiose	+	−	+	+
D-Galactose	−	−	v	+
Sucrose	−	+	+	+
Trehalose	+	−	v	+

[a] Abbreviations: −, None; d, colorless; w, white; y, yellow; o, orange; p, pink; b, brown; gy, gray; bl, blue.
[b] v: 11~89% of strains are positive.

glucose 0.5%, soybean meal 1%, peptone 0.5%, meat extract 1%, $CaCO_3$ 0.3% and sodium chloride 0.2%. Fermentation was carried out at 28°C on a rotary shaker at 250 rpm agitation. The antibiotic production was monitored by the paper-disc agar diffusion assay using *Bacillus subtilis* M45 (rec$^-$ mutant)[6] as the test organism. The antibiotic activity in the culture broth gradually increased and reached a maximum of 300 μg/ml after 6~7 days.

Fermentation was also carried out in a tank fermenter (200 liters). The second seed culture (5,000 ml) described above was inoculated to the fermenter containing 120 liters of the production medium composed of corn starch 5.0%, soybean meal 1.0%, Pharmamedia 1.0%, yeast extract 1.0% and $CaCO_3$ 1.0%, the pH was adjusted to 7.0 after sterilization. The tank fermenter was operated at 28°C at 250 rpm agitation and an aeration rate of 120 liters/minute. The pH of the culture broth gradually rose with the progress of fermentation and reached 7.8 after 72 hours when a peak antibiotic potency of 500 μg/ml was obtained.

Isolation and Purification

Extraction

The harvested broth (110 liters) was centrifuged and the filtrate was stirred with 5.5 liters of Trisacryl DEAE for 1 hour. The resin was washed with water (100 liters) and eluted three times with 5-liter portions of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.3 M sodium chloride. The eluates were combined, concentrated to 3 liters, and dialyzed against running water at 5°C overnight. The retentate solution contained *ca.* 54 g of crude maduropeptin based on lyophilization of a portion of the solution.

Isolation of Maduropeptins A_1, B, C

The above solution was applied to a Trisacryl DEAE column (4.0 × 80 cm) which had been pre-washed with 0.01 M Tris-HCl buffer (pH 7.4) at 5°C in a dark cold room. After washing with the buffer, the column was developed with the same buffer solution containing an increasing gradient of sodium chloride (0.1 M→0.15 M). The eluate was monitored by UV absorption at 210 nm, bioassay against *B. subtilis* M45,

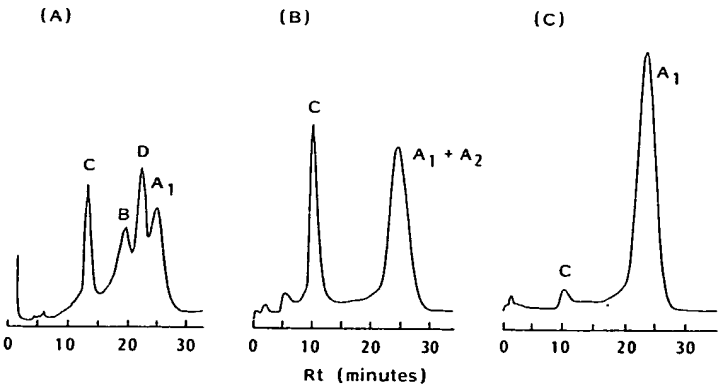
and HPLC (TSK Gel DEAE 3SW column, 0.01 M phosphate buffer pH 7.0 containing 0.13 M sodium sulfate elution). Four components, maduropeptin C (Rt 12.4 minutes), B (Rt 19.5 minutes), D (Rt 21.9 minutes) and A_1 (Rt 24.9 minutes) were eluted. Maduropeptins A_1, B and D were bioactive while C was bioinactive. The first, bioinactive, fractions were pooled, concentrated below 40°C and dialyzed against deionized water at 5°C in a dark room for 18 hours. Concentration of the retentate yielded a semi-pure solid of maduropeptin C. The second UV-absorbing fractions were pooled and worked up as above to yield a solution of semi-pure maduropeptins A_1, B and D mixture (estimated weight 4.78 g). One third of the solution was re-chromatographed on a column of Trisacyl DEAE (4.0 × 25 cm). Elution was carried out with 0.01 M Tris-HCl buffer (pH 7.4) containing sodium sulfate (starting from 0.05 M up to 0.15 M). Upon monitoring by bioassay and HPLC, two active peak fractions were eluted. They were concentrated and dialyzed to give solutions of maduropeptin A_1 (calcd weight 105 mg) and maduropeptin B (calcd weight 326 mg). The eluates between the above two peaks contained mostly maduropeptin D and small amount of maduropeptins A_1 and B. The semi-pure solution of maduropeptin A_1 (calcd weight 70 mg) was further purified by semi-preparative HPLC: column, TSK 545 DEAE (21.5 × 150 mm, LKB) and mobile phase, 0.01 M Tris-barbital buffer, pH 7.0 containing 0.2~0.25 M sodium sulfate (linear gradient). The eluate was monitored by HPLC and appropriate fractions were combined and dialyzed against deionized water to obtain a solution containing nearly pure maduropeptin A_1 (14 mg weight). The semi-pure maduropeptin B solution (70 mg equivalent) was similarly purified to yield a solution containing 27 mg of nearly pure maduropeptin B.

Purification of Photo-degradation Products

The natural maduropeptin components A_1, B, C and D, were found to be reasonably stable at 5°C in the dark but components B and D were shown by HPLC to be readily decomposed by light to a mixture of maduropeptins A_1 and C (Fig. 3). When the solution was allowed to stand at room temperature under light, maduropeptin A_1 was also decomposed giving only bioinactive maduropeptin C. Purification and characterization of the major products was attempted, utilizing photo-degradation to simplify the problem.

A solution of components A_1, B, C and D (ratio, 29 : 23 : 35 : 21) was gently stirred under a fluorescent

Fig. 3. HPLC chromatograms of maduropeptin mixture, photo-decomposed products and maduropeptin A_1.
(A) Maduropeptin mixture. (B) photo-decomposed products. (C) maduropeptin A_1.

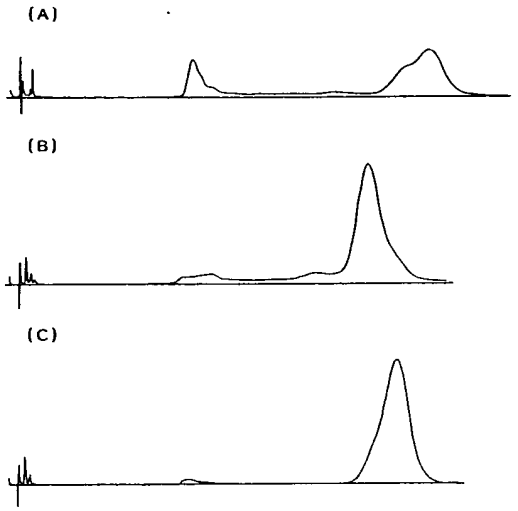


(A): Component ratio of A_1, 29; B, 23; C, 35; D, 21. (B): component ratio of $A_1 + A_2$, 62; C, 38. (C): component A_1 97% purity. HPLC column: TSK Gel DEAE 3SW (7.5 × 75 mm, Toyo Soda Manufacturing Co., Tokyo): solvent: 0.01 M phosphate buffer (pH 7.0) + 0.13 M Na_2SO_4; detection: UV (210 nm): flow rate: 1 ml/minute.

lamp (15 W) at 5°C for 65 hours. The resulting reaction solution appeared to contain only components A_1 and C (ratio 62 : 38) by HPLC analysis. The component A_1 in the solution was, however, found to be a mixture of maduropeptins A_1 and A_2 as described later. The solution was charged on a column of Trisacryl DEAE (2.0 × 95 cm). Elution was carried out first with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.11 M sodium chloride and then with a gradient of sodium chloride from 0.12 M to 0.15 M. The bioinactive UV fractions and bioactive UV fractions were pooled and desalted by dialysis to give maduropeptin C solution (calcd weight, 25 mg; purity, 99%) and A_1 solution (71 mg; purity, 90%).

Fig. 4. Preparative HPLC chromatograms of photo-decomposed maduropeptin, maduropeptins A_1 and A_2.

(A): Photo-decomposed maduropeptin (A_1+A_2+ C), (B) maduropeptin A_1, (C) maduropeptin A_2.



HPLC column: TSK Gel DEAE 3SW (7.5 × 75 mm, Toyo Soda Manufacturing Co., Tokyo), solvent: 0.01 M phosphate buffer (pH 7.0) + 0.09 M Na_2SO_4, detection: UV (210 nm), flow rate: 1 ml/minute.

Although the maduropeptin A_1 obtained showed single peak identical with that of natural maduropeptin A_1 by the above HPLC system, a modified HPLC system (0.01 M phosphate buffer, pH 7.0, containing 0.09 M sodium sulfate) revealed that it was a mixture of two components, the natural A_1 and a new component named A_2. Careful comparison of the photo-degradation products indicated that maduropeptins B and D were decomposed by light to yield component A_2. A part of the A_1 and A_2 mixture solution (39 mg) was subjected to semi-preparative HPLC (TSK Gel 545 DEAE, 21.5 × 150 mm, and 0.01 M phosphate buffer, pH 7.0, containing 0.25 M sodium chloride). The appropriate fractions were dialyzed against deionized water at 5°C to yeild component A_1 solution (2 mg, 97%) and component A_2 solution (6 mg, 99%) (Fig. 4).

Physico-chemical Properties

The four components, maduropeptins A_1, A_2, B and C were isolated as described above but maduropeptin D has not been obtained as a single entity. Maduropeptin C is very stable and A_1 and A_2 are fairly stable to isolation work-up. Maduropeptin B is, however, very unstable upon solidification and thus only limited data on this component were obtained. They are distinguished from each other by HPLC as shown in Fig. 3.

The four components are readily soluble in water but are insoluble and decompose in organic solvents resulting in complete loss of the activity. They were positive with Folin-Lowry, xanthoprotein, biuret and ninhydrin reagents but negative to Sakaguchi and anthrone reagents. Maduropeptins A_1 and A_2 exhibited UV absorption maxima at 210, *ca.* 280 and 308 nm while maduropeptin C lacked the maximum at 308 nm. Some of the physico-chemical data are shown in Table 4. The acidic nature of the four maduropeptin components was indicated by their isoelectric points. Maduropeptins A_1, A_2 and B are stable in neutral conditions at 5°C but gradually decompose at room temperature. They are unstable in acidic or alkaline solution or upon UV irradiation.

Table 4. Physico-chemical properties of maduropeptin components.

	Maduropeptin			
	A_1	A_2	B	C
Nature	White powder	White powder	White powder	White powder
MP (dec, °C)	240~244	226~230	235~238	249~252
$[\alpha]_D^{27}$	−84°	−27°	−48°	−76°
	(*c* 0.1, H_2O)	(*c* 0.2, H_2O)	(*c* 0.2, H_2O)	(*c* 0.5, H_2O)
Isoelectric point	4.75	4.90	4.80	4.77
Elementary analysis (Found)				
C:	45.07	48.13	43.91	46.97
H:	6.34	6.60	6.05	7.13
N:	12.70	13.18	12.66	13.82
S:	0.98	0.98	2.24	1.12
UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$)	210 (129), 286 (9.8), 308 (7.8)	201 (234), 278 (10.2), 306 (7.1)	275 (7.9)	219 (55), 278 (2.8)
IR ν_{max} (KBr) cm^{-1}	3400~3200, 1640, 1530	3450~3200, 1640, 1530	3450~3200, 1640, 1530	3450~3200, 1640, 1520
MW				
Gel filtration[a]				22,500
HPLC[b]				27,000

[a] Sephadex G-75.
[b] Asahipak GS-320.

MW Determination

The MW of maduropeptin C was estimated by gel filtration using a Sephadex G-75 column (22 × 685 mm) and 1/15 M phosphate buffer pH 7.0 with a flow rate of 30 ml/hour. The calibration standard kit (Pharmacia Fine Chem.) comprised of blue dextran (MW 2,000,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), chymotrypsinogen (MW 25,000) and ribonuclease A (MW 13,700) was chromatographed simultaneously. Maduropeptin C was eluted just after chymotrypsinogen as monitored by HPLC and its MW was calculated to be 22,500. Upon HPLC co-chromatography with the standard kit (Asahipak GS-320, 7.6 × 500 mm, Asahi Chemical Industry Co. and 0.1 M phosphate buffer, pH 7.0, containing 0.3 M sodium chloride), maduropeptin C was eluted before chymotrypsinogen indicating a molecular weight of 27,000.

Amino Acid Analysis

Maduropeptins A_1, B and C were hydrolyzed with 6 N HCl at 110°C for 22 hours. Part of the solution was oxidized with performic acid at 110°C for 20 hours for determination of cystine and tryptophan. After concentration to dryness *in vacuo*, the residue was subjected to amino acid analysis by a Waters Pico tag amino acid analyzer (Waters type ALC/GPC 606) with the results described in Table 5. Noteworthy is that all maduropeptin components do not contain basic amino acids, which distinguish them from the known macromolecular chromoprotein antibiotics.

Non-protein Chromophore

Maduropeptins B and D are highly sensitive to UV light yielding the bioinactive apoprotein maduropeptin C. Maduropeptins A_1 and A_2 are rather refractory to UV, but upon treatment with acidic methanol, they afforded a lipophilic substance with antimicrobial and cytotoxic activity, along with maduropeptin C. Preliminary characterization indicated that the bioactive degradation product has UV absorption considerably different from that of the original antibiotics and does not show synergistic

Table 5. Amino acid composition of maduropeptin components.

	Maduropeptin				Neocarzinostatin[a]
	A_1	A_2	B	C	
Lysine	—	—	—	—	0.96
Histidine	—	—	—	—	—
Arginine	—	—	—	—	1.60
Aspartic acid	6.0	5.2	5.8	5.4	10.00
Threonine	9.9	11.4	10.1	11.3	9.67
Serine	6.3	4.3	4.8	4.3	8.35
Glutamic acid	6.2	5.3	5.5	5.5	4.15
Proline	4.1	4.3	4.8	4.3	2.38
Glycine	9.3	7.9	7.7	8.2	12.15
Alanine	8.5	8.7	8.7	8.7	13.80
1/2 Cystine	—	0.4	0.9	0.3	3.10
Valine	7.4	6.8	11.1	7.1	8.90
Methionine	0.2	0.9	—	0.7	—
Isoleucine	1.7	1.0	1.0	1.8	0.94
Leucine	(1.0)[b]	(1.0)	(1.0)	(1.0)	5.03
Tyrosine	0.8	1.1	0.9	1.0	0.74
Phenylalanine	1.0	1.6	4.5	1.6	4.64
Tryptophan	—	—	—	—	1.64

[a] Literature values (J. Antibiotics, Ser. A 19: 253~259, 1966).
[b] Content of leucine is arbitrary assigned as 1.0.

Table 6. Antimicrobial activity of maduropeptin components and neocarzinostatin.

Test organisms	MIC (μg/ml)				
	A_1	A_2	B	C	Neocarzinostatin
Staphylococcus aureus 209P	1.6	1.6	1.6	>100	0.8
S. aureus Smith	0.8	1.6	0.8	>100	0.8
S. aureus D136	3.1	3.1	1.6	>100	1.6
S. epidermidis D153	3.1	3.1	1.6	>100	0.2
Micrococcus luteus PCI 1001	3.1	1.6	1.6	>100	1.6
Bacillus subtilis PCI 219	3.1	1.6	1.6	>100	1.6
Escherichia coli Juhl	>50	>50	>25	>100	>25
Klebsiella pneumoniae D11	>50	>50	>25	>100	>25
Pseudomonas aeruginosa A9930	>50	>50	>25	>100	>25
Proteus vulgaris A9436	>50	>50	>25	>100	>25
Candida albicans A9540	>50	>50	>25	>100	>25
Cryptococcus neoformans D49	>50	>50	>25	>100	>25
Aspergillus fumigatus IAM 2530	>50	>50	>25	>100	>25
Trichophyton mentagrophytes D155	>50	>50	>25	>100	>25

antimicrobial effect with maduropeptin C[7]. The results suggested that the bioactive degradation product was not a true non-protein chromophore but rather an artifact.

Antimicrobial Activity

The antimicrobial activity, measured as MIC, of maduropeptins A_1, A_2, B and C was assessed using the agar dilution assay. Nutrient broth was used for Gram-positive and Gram-negative bacteria and Sabouraud dextrose broth for fungi. The inoculum size was adjusted to $10^5 \sim 10^6$ cfu/ml for bacteria and 10^6 cfu/ml for fungi. Incubation was carried out at 28°C for 18 hours and neocarzinostatin (NCS) was used as a reference compound. The results are shown in Table 6.

Maduropeptins A_1, A_2 and B exhibited significant inhibitory activity against Gram-positive bacteria with MICs being 2~15 times greater than those of NCS. They were inactive against Gram-negative bacteria. Maduropeptin C, the apoprotein of the components A_1, A_2 and B, did not show *in vitro* activity.

Antitumor Activity

Maduropeptin components were tested for *in vitro* cytotoxicity against murine and human tumor cells and for *in vivo* antitumor activity in mice. NCS was used as a reference compound for both *in vitro* and *in vivo* experiments.

In Vitro Cytotoxicity

Murine melanoma B16-F10 cells were grown in EAGLE's minimum essential medium supplemented with fetal calf serum (FCS, 10%) and kanamycin (60 μg/ml), and human colon carcinoma HCT-116 cells in McCOY's 5A medium supplemented with FCS (10%), benzylpenicillin (100 U/ml), and streptomycin (100 μg/ml). Exponentially growing B16-F10 and HCT-116 cells were harvested, counted and suspended in the culture medium at 3×10^4 and 6×10^4 cells/ml, respectively. After planting 180 μl of cell suspension into wells of a 96-well microtiter plate with test samples (20 μl), the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 72 hours. The cytotoxicity was colorimetrically determined at 540 nm after staining the viable cells with neutral red solution. Maduropeptin components A_1, A_2 and B showed potent cytotoxicity with IC_{50} values ranging from 0.007 to 0.16 μg/ml against both tumor cells. In particular, component A_1 gave 65-fold (vs. B16-F10) and 190-fold (vs. HCT-116) more potent activity than NCS. Maduropeptin C did not show cytotoxicity at 100 μg/ml against either cell line (Table 7).

Table 7. *In vitro* cytotoxicity against murine melanoma B16 and human colon carcinoma HCT-116 cells.

Compound	IC_{50} (μg/ml)	
	B16-F10	HCT-116
Maduropeptin A_1	0.017	0.007
Maduropeptin A_2	0.043	0.16
Maduropeptin B	0.028	0.029
Maduropeptin C	>100	>100
Neocarzinostatin	1.1	1.3

In Vivo Antitumor Activity

The *in vivo* antitumor activity of maduropeptin

Table 8. Antitumor activity of maduropeptin components against P388 (ip) in female CDF_1 mice.

Compound	Treatment (QID × 3, ip) mg/kg/day	MST (day)	T/C (%)	Average weight change on day 4 (g)
Maduropeptin A_1	0.06	16.5	165	−1.3
	0.02	15.0	150	−0.5
	0.006	14.0	140	0.0
	0.002	11.0	110	+1.0
Maduropeptin A_2	0.06	7.0	70	−1.0
	0.02	15.5	155	−1.0
	0.006	15.0	150	−0.5
	0.002	14.0	140	+0.5
	0.0006	11.0	110	+1.8
Maduropeptin B	1	7.0	70	−2.3
	0.3	23.5	235	−3.0
	0.1	16.0	160	−2.0
	0.03	15.5	155	−0.8
	0.01	14.0	140	0.0
Vehicle	—	10.0	—	+0.8

Table 9. Antitumor activity of maduropeptin A_1 against P388 (ip) in male BDF_1 mice.

Compound	Treatment (Q1D × 9, ip) mg/kg/day	MST (day)	T/C (%)	Average weight change on day 4 (g)
Maduropeptin A_1	0.025	9.0	90	−1.8
	0.013	18.0	180	−1.7
	0.006	18.0	180	−0.3
	0.003	16.0	160	0.0
	0.0016	15.0	150	+1.2
	0.0008	14.0	140	+1.2
Neocarzinostatin	1	13.5	135	−0.8
	0.5	21.0	210	+0.3
	0.25	19.0	190	+1.0
	0.13	18.5	185	+1.2
	0.063	18.5	185	+1.8
	0.031	17.0	170	+1.8
	0.016	14.5	145	+1.2
	0.008	14.0	140	+1.5
Vehicle	—	10.0	—	+2.1

Table 10. Antitumor activity of maduropeptin A_1 against B16 melanoma in male BDF_1 mice (subrenal capsule assay).

Compound	Dose (Q1D × 5, ip) mg/kg/day	Δ Tumor size (OMU[a] ±SE)	% Inhibition of tumor growth
Maduropeptin A_1	0.02	0.3±1.3	97
	0.01	1.6±2.2	86
	0.005	5.3±3.2	53
	0.0025	10.6±5.5	6
Vehicle	—	11.3±2.9	—
Neocarzinostatin	1.0	3.5±2.1	84
	0.5	7.1±1.9	67
	0.25	9.2+1.9	57
	0.13	14.8±2.6	31
Vehicle	—	21.3±1.9	—

[a] Ocular micrometer units.

components was examined against P388 lymphocytic leukemia and melanoma B16. P388 cells (10^6 cells per mouse) were inoculated intraperitoneally into male BDF_1 or female CDF_1 mice. Graded doses of the test materials were administered intraperitoneally to groups of 4 female CDF_1 mice on days 1 to 3 (Q1D × 3) or to groups of 6 male BDF_1 mice on days 1 to 9 (Q1D × 9) after tumor implantation (day 0). Death or survival of the treated and non-treated animals was recorded daily during the observation period of 45 days and the median survival time (MST) was calculated for the test (T) and control (C) groups. A T/C value of $\geqq 125\%$ is considered a significant antitumor effect. As shown in Table 8, maduropeptin components A_1, A_2 and B gave highly potent antitumor activity with maximum T/C values of 165%, 155% and 235% (Q1D × 3 treatment), respectively, against P388 leukemia. When compared with NCS in the Q1D × 9 treatment against P388 leukemia, maduropeptin A_1 was approximately 10-fold more potent than NCS in terms of minimum effective dose (Table 9). Anti-B16 melanoma activity of maduropeptin A_1 was determined

in the mouse subrenal capsule (SRC) assay[8]. A minced fragment of the melanoma was implanted beneath the renal capsule of male BDF_1 mice and each initial graft size was measured with an ocular micrometer scale (day 0). The animals were randomized in groups of 4 and treated with graded doses of the test materials on days 1 to 5 (Q1D × 5). On day 6, the kidney was excised and the final graft size was determined. Antitumor activity was expressd as % inhibition of tumor growth and an inhibition value of $\geqq 50\%$ is considered significant antitumor activity. As shown in Table 10, maduropeptin A_1 gave dose-related inhibition of tumor growth and was approximately 50-fold more active than NCS in terms of minimum effective dose.

Acute Toxicity

The acute toxicity was determined by intraperitoneal administration of graded doses of the materials to groups of 5 normal male *dd*Y mice. The LD_{50} was calculated 10 days after administration according to the method of VAN DER WAERDEN[9]. Maduropeptin A_1 (LD_{50} 0.067 mg/kg) was approximately 50-fold more toxic than NCS (LD_{50} 3.1 mg/kg) tested as a reference.

Discussion

Five components of antitumor antibiotic maduropeptin have been isolated from the culture filtrate of *A. madurae* strain No. H710-49. They are acidic polypeptides and the bioactive components, maduropeptins A_1, A_2, B and D carry a chromophore unit.

Many chromoprotein antibiotics including neocarzinostatin[10], auromomycin[11], macromomycin[12], actinoxanthin[13], sporamycin[14], largomycin[15] and C-1027[16] have been identified as effective antitumor agents and, among them, neocarzinostatin is in clinical use. Maduropeptin appears to be a new addition to this family from its physico-chemical and biological profile, but it is distinctly different from the preceding antibiotics in not containing basic amino acids. As mentioned previously, attempts to isolate a true non-protein chromophore of maduropeptin were unsuccessful. Isolation and characterization of the chromophore are continuing and will be reported later.

Acknowledgments

The authors are grateful to Dr. T. OKI, the director of their institute for his encouragement and helpful discussion.

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EXHIBIT G

PRADIMICINS A, B AND C†: NEW ANTIFUNGAL ANTIBIOTICS

I. TAXONOMY, PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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New antifungal antibiotics, pradimicins A, B and C were isolated from the culture broth of actinomycete strains proposed as *Actinomadura hibisca*. They are orange to red pigments containing a benzo[*a*]naphthacenequinone chromophore substituted with a D-alanine, an aminosugar and a D-xylose (pradimicins A and C).

In a systematic search for microbial metabolites effective against fungi and yeasts, actinomycete strains No. P157-2 and Q278-4 isolated from soil samples collected in the Fiji Islands and India, respectively, were found to produce a complex of novel antibiotics designated pradimicin[1~3]. The producing strains were classified as an undescribed species of the genus *Actinomadura* and named *Actinomadura hibisca* sp. nov. after taxonomical studies. The active principle was precipitated from the broth filtrate at pH 5.0 and purified by solvent partition and column chromatography to yield a major component pradimicin A and two minor components pradimicins B and C. Pradimicins A, B and C showed moderate *in vitro* activity against a wide variety of fungi and yeasts, some Gram-positive bacteria, and viruses. Most interestingly, they exhibited marked *in vivo* therapeutic activity against systemic fungal infections caused by *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* strains in mice. Through chemical degradation and spectral analyses, the structures of pradimicins have been determined to be a benzo[*a*]naphthacenequinone carrying D-alanine and sugars[4~6]. In this paper, we report the taxonomy of the producing strains and the production, isolation and physico-chemical properties of pradimicins. The biological activities of the antibiotics are reported in a companion paper[7].

Taxonomy of the Producing Strain

The producing organisms, strain P157-2 and strain Q278-4 were isolated from soil samples collected in the Fiji Islands and Andhra Pradesh State, India, respectively.

Morphological Characteristics

Both strains form white aerial mycelium and non-fragmentary substrate mycelium. Long straight spore-chains (10 to 50 spores per chain) are formed on all parts of the aerial mycelium. The spores are oblong in shape, 0.4 to 0.6 by 0.7 to 1.5 μm in size, non-flagellate, and have smooth surface (Fig. 1). A fused coil of spore chain is occasionally formed intercalary or at the tip of long chains, which is observed by photomicroscopy as a small globose body (1.5 to 4 μm in diameter) (Fig. 2).

† Pradimicin A was originally called as BU-3608 A or BMY-28567, pradimicin B as BU-3608 B or BMY-28634 and pradimicin C as BU-3608 C or BMY-28747.

Fig. 1. Scanning electron micrograph of spore chains of strain P157-2 on inorganic salts-starch agar (ISP medium No. 4) incubated at 28°C for 14 days.

Bar represents 3 μm.

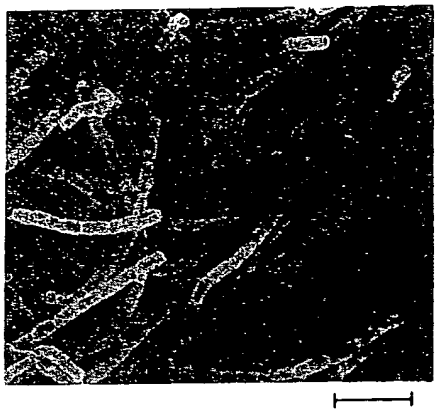
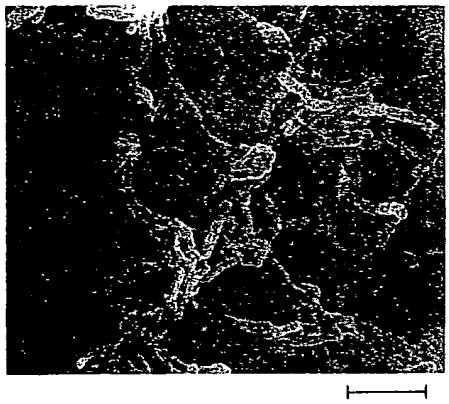


Fig. 2. Scanning electron micrograph of spore chains with intercalary globose body, of strain P157-2 on yeast extract-malt extract agar (ISP medium No. 2) incubated at 28°C for 14 days.

Bar represents 3 μm.

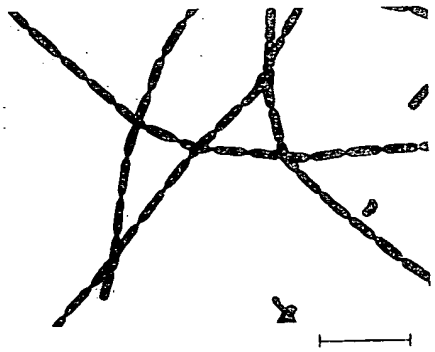


Cultural and Physiological Characteristics

White aerial mycelium is formed on some media such as yeast extract-malt extract agar (ISP medium No. 2) (Fig. 3). A reddish pigment, which is recognized as pradimicins, is produced abundantly in ISP media No. 2 and 6, but slightly in ISP media No. 3, 4 and 5, and a brown melanoid pigment only in ISP medium No. 7. The growth temperature range is 18 to 40°C for strain P157-2 and 16 to 43°C for strain Q278-4. The NaCl tolerance for growth is seen at 3% (w/v) but not at 6% (w/v). The cultural characteristics of strains P157-2 and Q278-4 are shown in Table 1, and the physiological characteristics examined by the methods of GORDON *et al.*[8] are given in Table 2.

Fig. 3. Transmission electron micrograph of spore chains of strain Q278-4 on yeast extract-malt extract agar (ISP medium No. 2) at 28°C for 14 days.

Bar represents 5 μm.



Chemotaxonomical Studies

The whole-cell hydrolysate contains *meso*-diaminopimelic acid, glucose and a small amount of madurose, hence the cell wall is type III and the whole cell-sugar is Pattern B. The phospholipids contain phosphatidylglycerol and phosphatidylinositol but lack nitrogenous phospholipids, hence belong to type P-I. The major menaquinone is MK-9 (H_6). Glycolate test is negative. Mycolate is absent.

Taxonomic Position

Based on the major characteristics of strains P157-2 and Q278-4, both strains were placed in the genus *Actinomadura*. Many strains of *Actinomadura* produce reddish pigments such as those identified as prodigiosins or anthracyclines, while these two strains produce three new red pigments, pradimicins A, B and C having benzo[*a*]naphthacenequinone as aglycone.

Mediu

Sucrose-nitrate (CZAPEK-Do:

Yeast extract-m extract agar (I

Glycerol-asparag (ISP No. 5)

Glucose-asparagi

Tyrosine agar (ISP No. 7)

Observations aft
Abbreviations: G
Color name used

Table 2.

Decompositic
Adenine
Casein
Hypoxanth
Tyrosine
Urea
Xanthine
Resistance to:
Lysozyme
Hydrolysis of:
Aesculin
Hippuric aci
Starch
Acid from:
Adonitol
L-Arabinose
Cellobiose
Erythritol
Glucose
Glycerol
Inositol
Lactose
Mannitol

Abbreviations:
+: 85 to 100%
The data of *A.*

Table 1. Cultural characteristics of strains P157-2 and Q278-4.

Medium	Strain P157-2	Strain Q278-4
Sucrose-nitrate agar (CZAPEK-Dox agar)	G: Scant A: None S: Pinkish white (9)	G: Poor A: None S: Colorless to pale purplish pink (252)
Yeast extract-malt extract agar (ISP No. 2)	D: Pale yellowish pink (31) G: Moderate A: Moderate; white S: Very deep red (14) D: Very dark red (17)	D: Pale purplish pink (252) G: Good A: Moderate; white S: Deep red (13) D: Very dark red (17)
Glycerol-asparagine agar (ISP No. 5)	G: Poor A: Scant; white S: Colorless to light pink (4) D: Moderate yellowish pink (29)	G: Poor A: None S: Light grayish red (18) D: Light yellowish pink (28)
Glucose-asparagine agar	G: Abundunt A: None S: Very deep red (14)	G: Moderate A: None S: Dark red (16), later dark grayish purple (229)
Tyrosine agar (ISP No. 7)	D: Very dark red (17) G: Poor A: Very scant; white S: Light brown (57) D: Deep brown (59)	D: Grayish purplish pink (253) G: Poor A: Poor; white, later light gray (264) S: Dark reddish brown (44) D: Dark grayish reddish brown (47)

Observations after incubation at 28°C for 3 weeks.
Abbreviations: G, growth; A, aerial mycelium; S, substrate mycelium; D, diffusible pigment.
Color name used: ISCC-NBS Color-Name Charts.

Table 2. Physiological characteristics of *Actinomadura hibisca* and *Actinomadura pelletieri*.

	A.h.	*A.p.*		*A.h.*	*A.p.*
Decomposition of:			Mannose	−	−
Adenine	+	−	Melezitose	−	−
Casein	+	+	Melibiose	−	−
Hypoxanthine	+	+	Methyl α-glucoside	−	−
Tyrosine	+	+	Raffinose	−	−
Urea	−	−	L-Rhamnose	−	−
Xanthine	−	−	Sorbitol	−	−
Resistance to:			Trehalose	+	+
Lysozyme	+	−	D-Xylose	−	−
Hydrolysis of:			Utilization of:		
Aesculin	+	−	Benzoate	−	−
Hippuric acid	−	−	Citrate	−	−
Starch	−	−	Mucate	−	−
Acid from:			Succinate	v	−
Adonitol	−	−	Tartrate	−	−
L-Arabinose	−	−	Production of:		
Cellobiose	+	−	Gelatinase	+	+
Erythritol	−	−	Nitrate reductase	+	+
Glucose	+	+	Melanoid	+	v
Glycerol	−	−	Prodigiosin	−	+
Inositol	−	−	Collagenase	−	+
Lactose	−	−	Tyrosinase	−	−
Mannitol	−	−			

Abbreviations: *A.h.*, *Actinomadura hibisca*; *A.p.*, *A. pelletieri*.
+: 85 to 100% of strains positive. −: 0 to 14% of strains positive. v: 15 to 84% strains positive.
The data of *A. pelletieri* are cited from the literature of GORDON *et al.*[8].

The chemotaxonomy[9] and numerical taxonomy[10,11] of *Actinomadura* as well as the descriptions of many new species of the genus such as *Actinomadura atramentaria*[12] indicate that the two strains have some similarity to *Actinomadura pelletieri*. However, they are differentiated from *A. pelletieri* in the ability to form long spore-chains, the decomposition of adenine, the resistance to lysozyme, the hydrolysis of aesculin, the acid formation from cellobiose, and the production of pradimicins instead of prodigiosin of *A. pelletieri* (Table 2). Thus, strains P157-2 and Q278-4 are considered to be a new species of *Actinomadura* and are proposed to name *Actinomadura hibisca* sp. nov. TOMITA (Origin: hi-bisika, L.n.; Gr *hibiskos* rose mallow, a plant with reddish flower reffering to the red diffusible pigments. The type strain is No. P157-2 (ATCC 53557).

Antibiotic Production

A slant culture of *A. hibisca* strain P157-2 (ATCC 53557) or strain Q278-4 (ATCC 53646) was prepared using modified BENNETT's agar medium consisting of soluble starch 0.5%, glucose 0.5%, fish meal extract 0.1%, yeast extract 0.1%, NZ-case (Sheffield) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.6%, and incubated at 28°C for 7 days. A portion of the microbial growth from the slant culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of glucose 1%, soluble starch 2%, NZ-amine A (Sheffield) 0.5%, yeast extract 0.5% and $CaCO_3$ 0.1%, adjusted to pH 7.2 before sterilization. The seed culture was incubated at 28°C for 4 days on a rotary shaker (200 rpm); 5 ml of the culture was transferred to a 500-ml Erlenmeyer flask which contained 100 ml of fermentation medium consisting of glucose 3%, soybean meal 3%, Pharmamedia 0.5%, yeast extract 0.1% and $CaCO_3$ 0.3%. The fermentation was carried out at 28°C for 5 to 6 days on a rotary shaker.

Antibiotic production in the cultured broth was determined by the conventional broth dilution method using *C. albicans* A9540 as the indicator organism in Sabouraud dextrose medium. The visible absorption at 500 nm in 0.02 N NaOH-MeOH (1:1) solution was used in parallel with the microbial assay. The

Table 3. Physico-chemical properties of pradimicins A, B and C.

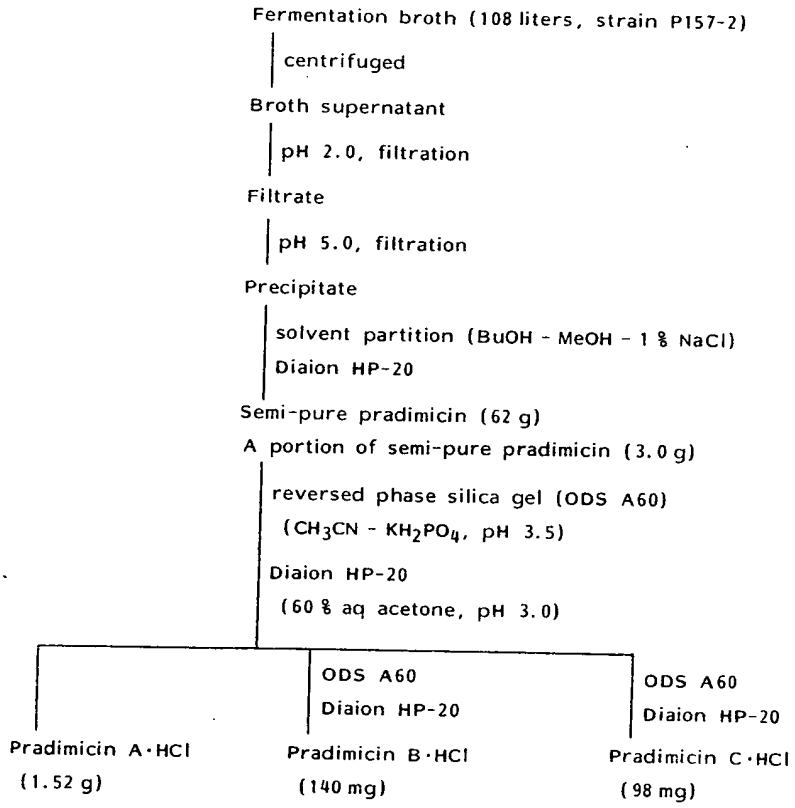
	Pradimicin A	Pradimicin B	Pradimicin C
Nature	Red amorphous powder	Red amorphous powder	Red amorphous powder
MP (°C, dec)	193~195	195~198	220~225
$[\alpha]_D^{26}$ (*c* 0.1, 0.1 N HCl)	+685°	+440°	+619°
UV and visible $\lambda_{max}^{50\% MeOH}$ nm (ε)	231 (28,300), 284 (22,700) 482 (9,600)	234 (27,900), 278 (23,100), 492 (8,800)	230 (31,400), 285 (23,400), 481 (9,900)
SI-MS *m/z*: Glycerol matrix	843 $(M+3H)^+$	711 $(M+3H)^+$	
mNBA matrix	841 $(M+H)^+$	709 $(M+H)^+$	827 $(M+H)^+$
Molecular formula	$C_{40}H_{44}N_2O_{18}$	$C_{35}H_{36}N_2O_{14}$	$C_{39}H_{42}N_2O_{18}$
TLC[a] Rf	0.36	0.48	0.32
HPLC[b] Rt (minutes)	16.7	21.8	14.0

[a] Silica gel 60 (Merck, 5715), MeOAc-1-PrOH-28% NH_4OH (45:105:60).
[b] Column: Microsorb Short One C_{18} (4.6 mm i.d. × 100 mm, Rainin Instrument), mobile phase: CH_3CN-0.15% KH_2PO_4 adjusted to pH 3.5 with H_3PO_4 (7:17), flow rate: 1.2 ml/minute, detection: UV absorption at 254 nm.

Table 4. Production ratio of pradimicins A, B and C.

Producing strain	Potency (μg/ml)	Production ratio (%)		
		Pradimicin A	Pradimicin B	Pradimicin C
Actinomadura hibisca P157-2	820	86.5	6.6	6.9
A. hibisca Q278-4	780	56.6	6.9	36.5

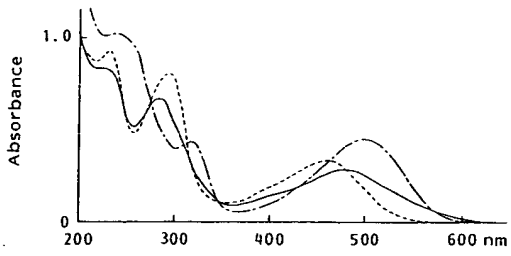
Fig. 4. Isolation and purification of pradimicin components.



antibiotic potency reached a maximum of 650 μg/ml on the 5th day. The fermentation was also carried out in a 200-liter tank fermenter. Three liters of the seed culture were used to inoculate 120 liters of production medium consisting of glucose 3%, Protein S (Ajinomoto Co.) 3% and $CaCO_3$ 0.3%. The tank fermenter was operated at 28°C with agitation at 250 rpm and an aeration rate of 120 liters/minute. The pH of the cultured broth gradually rose with the progress of fermentation and reached 7.9 after 96 hours. The antibiotic potency was 820 μg/ml.

Fig. 5. UV and visible spectrum of pradimicin A (25 μg/ml).

—— in 50% MeOH, ------ in 0.01 N HCl - 50% MeOH, —·— in 0.01 N NaOH - 50% MeOH.



Strain P157-2 produced pradimicin A as a major component and a small amount of pradimicins B and C in the broth; strain Q278-4 produced pradimicins A and C as major components with a small amount of pradimicin B.

The pradimicin components in the broth were analyzed by HPLC. The HPLC mobility of each component is shown in Table 3. An example of production ratio of pradimicins A, B and C in the tank fermentation using strains P157-2 and Q278-4 is shown in Table 4.

Fig. 6. IR spectrum of pradimicin A (KBr).

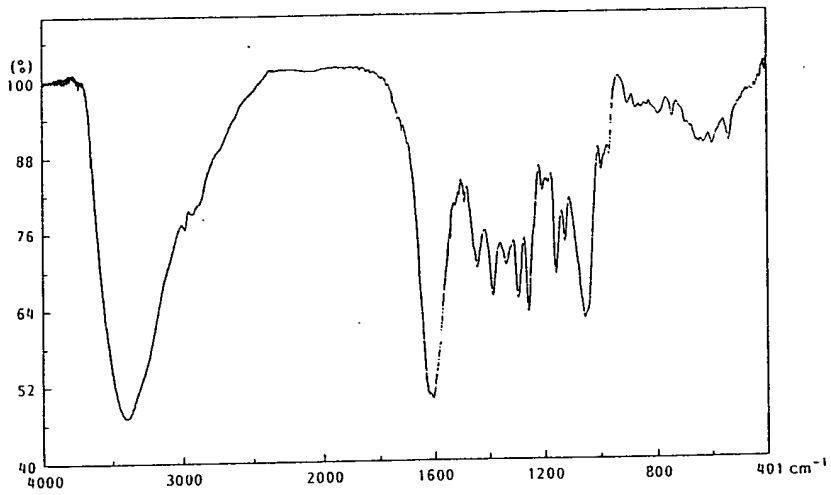
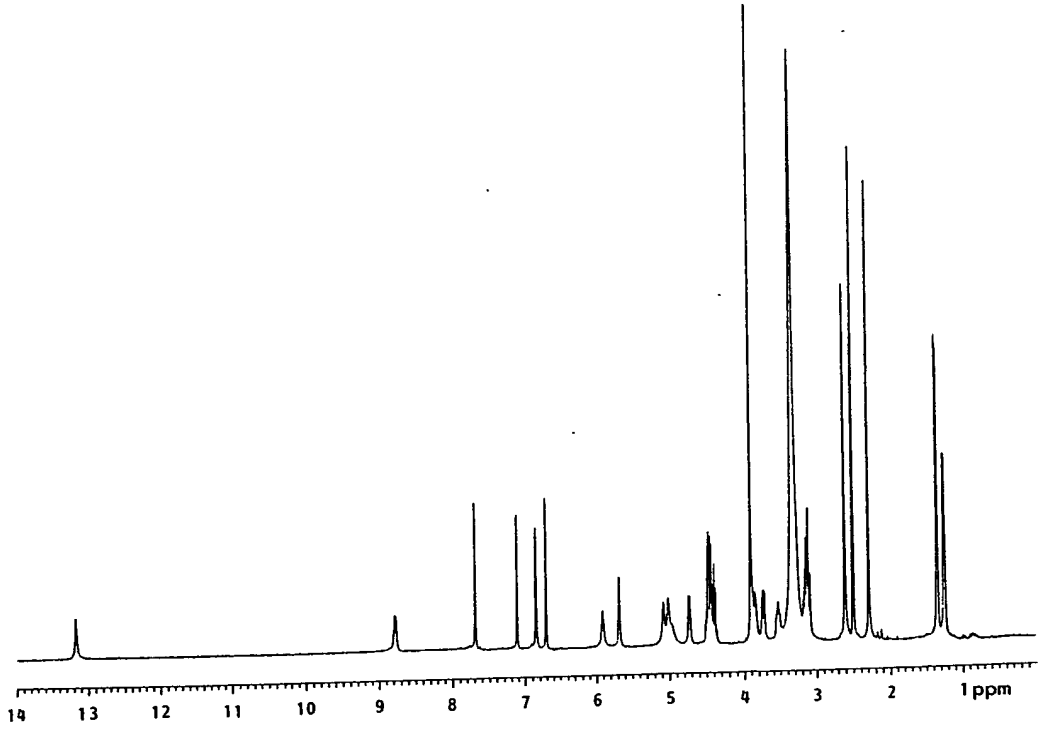


Fig. 7. ^{1}H NMR spectrum of pradimicin A (400 MHz, in DMSO-d_6).

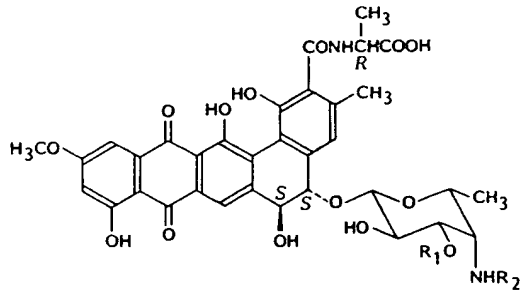


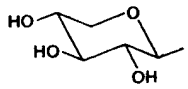
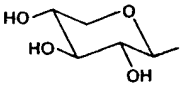
Isolation and Purification

The isolation procedure for the pradimicin components is summarized in Fig. 4 and the details are described in the previous paper[5].

Crystallization of the purified pradimicin A·HCl from MeOAc-1-PrOH-0.1 N NaOH gave fine needles of the monosodium salt of the antibiotic. When the salt was dissolved in water and adjusted to pH 5.0 by 0.1 N HCl, a precipitate of the zwitterionic form of pradimicin A deposited. Aqueous solution of pradimicin B·HCl and pradimicin C·HCl were similarly treated with 0.1 N NaOH to obtain the

Fig. 8. Structures of pradimicins A, B and C.



	R_1	R_2
Pradimicin A		CH_3
Pradimicin B	H	CH_3
Pradimicin C		H

zwitterionic forms.

Physico-chemical Properties

Zwitterionic pradimicins A, B and C exhibited the physico-chemical properties as summarized in Table 3. They are soluble in *N,N*-dimethylformamide, dimethyl sulfoxide and acidic and alkaline water, slightly soluble in water, methanol, ethanol and 1-butanol but insoluble in common organic solvents. The UV and visible spectrum and IR spectrum of pradimicin A are shown in Figs. 5 and 6, respectively. The CD spectrum of pradimicin A ($\lambda_{extreme}^{0.01\,N\,HCl}$ nm ($\Delta\varepsilon$) 213 (+1.9), 244 (−17.7), 289 (+15.3), 335 (−6.1), 515 (+6.6)) corresponded well with that of its aglycone reported before[4,5]. Fig. 7 shows the ^{1}H NMR spectrum of pradimicin A. As has been reported in a separate paper[4,5], the structures of pradimicins A, B and C were determined to have a benzo[*a*]naphthacenequinone nucleus substituted with D-alanine and sugars (Fig. 8).

Discussion

Novel antifungal antibiotics pradimicins A, B and C were discovered as the metabolites of a new species of the genus *Actinomadura*, later named *Actinomadura hibisca* sp. nov. Pradimicins A, B and C are red amorphous powders having characteristic UV absorption. The structural studies have revealed that they share the same unique aglycone, a dihydro benzo[*a*]naphthacenequinone nucleus substituted with D-alanine, and differ from each other in the type and number of sugar substituents (Fig. 8). As discussed in the following paper, pradimicins A, B and C demonstrated moderate *in vitro* activity against various clinically important fungi and yeasts and impressive *in vivo* activity against those fungal infections.

Recently, a complex of closely related antibiotics, benanomicin, was reported[13~15]. Benanomicin B is considered to be identical with pradimicin C from the reported data.

Acknowledgments

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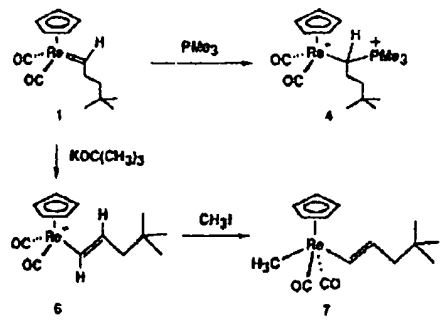

perature protonation of 6 in CH_3OD led to regeneration of carbene complex 1, which subsequently underwent acid-catalyzed isomerization to 2 at room temperature. Unlike other anions of electrophilic carbene complexes, which undergo C-alkylation, 6 is alkylated at rhenium.[25] For example, reaction of CH_3I with 6 produces *trans*-$C_5H_5(CO)_2(CH_3)Re[(E)$-$CH{=}CHCH_2C(CH_3)_3]$ (7).[12] The tendency of 6 to undergo alkylation at rhenium is related to the stability of $C_5H_5(CO)_2ReR_2$ systems.[26]

In summary, 1 is only the second example of a carbene complex that reacts with both nucleophiles and electrophiles at the carbene carbon atom. Derivatives of 1 are being synthesized to modulate the reactivity of the Re=C multiple bond.

Acknowledgment. Support from the National Science Foundation is gratefully acknowledged. P.C.V. thanks BP America for a fellowship.

Supplementary Material Available: Full spectral characterization of compounds 1-7 (3 pages). Ordering information is given on any current masthead page.

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Crystal and Molecular Structure of Dynemicin A: A Novel 1,5-Diyn-3-ene Antitumor Antibiotic

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The antitumor antibiotics of the esperamicin[1] and calicheamicin[2] families have aroused considerable interest because of their exceptional potency,[3] the structural novelty of their 1,5-diyne-3-ene core, and their intriguing mode of action.[4] In this paper, we report

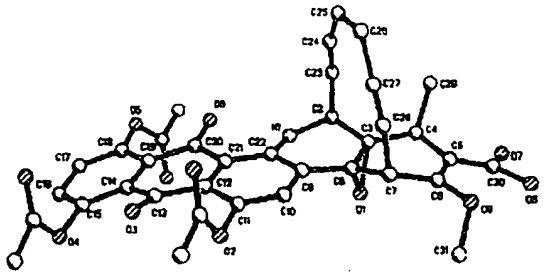
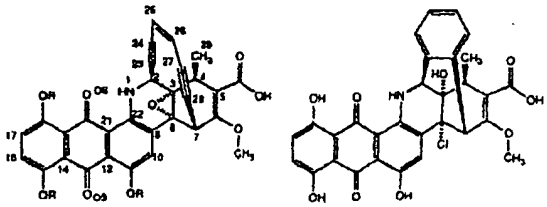


Figure 1. A computer-generated perspective drawing of the final X-ray model of dynemicin A triacetate (2). Hydrogens are omitted for clarity, and no absolute configuration is implied.

the structure of another 1,5-diyn-3-ene antibiotic: dynemicin A (1) from *Micromonospora chersina*.[5] Dynemicin A (1) has potent inhibitory activity against a wide range of bacteria and tumor cell lines, a structurally novel fusion of an anthraquinone with a tetracyclo 1,5-diyn-3-ene, and a putative mode of action similar to the esperamicins and calicheamicins.



1 R=H
2 R=$COCH_3$

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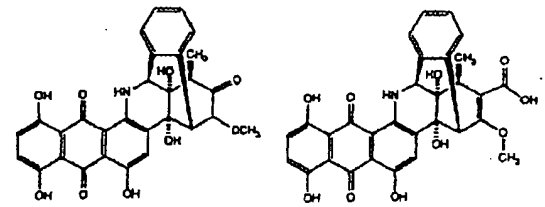

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Dynemicin A was isolated[5] from the ethyl acetate extract of *M. chersina* as a lipophilic violet solid: HRMS *m/z* 538.1132 $(M + H)^+$; $C_{30}H_{19}NO_9 + H$ requires 538.1138; mp 208-210 °C dec; $[\alpha]_D$ +270° (*c* 0.01, DMF); IR (KBr) 3420, 3350, 2930, 1660, 1630, 1587, 1480, 1385, 1300, 1280, 1180, and 785 cm^{-1}; UV (MeOH) λ_{max} 239 (ε 24900), 282 (sh), 569 (10800), and 599 nm (10100). The UV spectrum and the absorption shifts observed in weakly acid and alkaline solution[6] suggested a 1,4,5,8-tetrahydroxyanthraquinone chromophore. Poor solubility hampered spectral characterization of 1,[7] but conversion to its triacetate 2 (acetic anhydride–pyridine, 25 °C) provided a more tractable material. The ^{13}C NMR spectrum of 2 revealed a 1,2,4,5,8-pentasubstituted anthraquinone (δ_C 146.9, s, C18; 130.6, d, C17; 131.0, d, C16; 146.4, s, C15; 125.9, s, C14; 180.6, s, C13;

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(6) UV: λ_{max} in 0.01 N HCl–MeOH 240 (ε 26300), 284 (sh), 572 (10100), and 596 nm (10300); in 0.01 N NaOH–MeOH 247 (ε 22600), 278 (5400), 598 (11500), and 644 nm (11300). See: Oki, T.; Yoshimoto, A.; Matsuzawa, Y.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1988**, *33*, 1331-1340.

(7) ^1H NMR of 1 (400 MHz in DMSO d_6): δ 1.30 (3 H, d, J = 7.3), 3.57 (1 H, q, J = 7.3), 3.82 (3 H, s), 4.89 (1 H, s), 5.08 (1 H, d, J = 4.3), 6.06 and 6.09 (AB quartet, J = 9.8), 7.33 (1 H, d, J = 8.9), 7.38 (1 H, J = 8.9), 8.03 (1 H, s), 9.86 (1 H, d, J = 4.3), 12.15 (1 H, br s), 12.30 (1 H, v br s), 12.70 (1 H, br s), 13.10 (1 H, br s).

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124.5, s, C12; 139.5, s, C11; 130.0, d, C10; 130.1, s, C9; 143.8, s, C22; 114.7, s, C21; 182.7, s, C20; 126.1, s, C19) as well as a 1,5-diyn-3-ene system (δ_C 97.3, s, C23; 89.6, s, C24; 124.4, d, C25; 124.0, d, C26; 88.8, s, C27; 99.4, s, C28).[8] Triacetate 2 was further characterized by single-crystal X-ray diffraction.

The dark orange crystals of 2 belonged to the monoclinic space group $P2_1$ with a = 9.238 (4) Å, b = 13.047 (5) Å, c = 28.254 (9) Å, and β = 98.62 (3)°. The crystal density required that *two* molecules of triacetyldynemicin A (2) form the asymmetric unit (Z = 4). All unique diffraction maxima with $2\theta \leq 100°$ were collected by using variable-speed Wyckoff scans and graphite-monochromated Cu K$\bar{\alpha}$ radiation. Of the 3403 independent reflections surveyed in this manner, 2684 (79%) were judged observed ($|F_o| \geq 4.0\sigma(F_o)$) after correction for Lorentz, polarization, and background effects. A phasing model was found with some difficulty, and successive electron density syntheses finally revealed the entire nonhydrogen atom structure. Some hydrogen atoms were located in subsequent difference electron density syntheses, but the majority were included at appropriate locations. Blocked full-matrix least-squares refinements with anisotropic heavy atoms and fixed isotropic hydrogens have converged to a standard crystallographic residual of 8.4% for the observed data.

A computer-generated perspective drawing of the final X-ray model of triacetyldynemicin A (2) is given in Figure 1. The X-ray experiment defined only the relative, not the absolute configuration, and the enantiomer shown was selected arbitrarily. Attempts to prepare heavy-atom derivatives of dynemicin in order to determine the absolute configuration via anomalous dispersion techniques have not yet succeeded. There are two independent molecules in the asymmetric unit, and while they have the same overall structure, their conformations are distinctly different. In the molecule shown, the anthraquinone ring is bowed so that O3 and O6 are pushed up, on the same side of the molecule as the 1,5-diyn-3-ene moiety. The anthraquinone fragment is bowed so that there is a shallow concave surface on the bottom of the molecule, on the side away from the 1,5-diyn-3-ene moiety. In the other independent molecule, the bowing of the anthraquinone occurs in the opposite sense; O3 and O6 are on the bottom, as is the convex face of the anthraquinone. The bowing may be described more quantitatively by considering the dihedral angles between the right- and left-hand aromatic rings of the anthraquinone. In one conformation, it is 12°, and in the other, 17° in the opposite sense.

This X-ray structure of triacetyldynemicin A represents the first time that a naturally occurring 1,5-diyn-3-ene has been characterized by X-ray diffraction, but the geometry is similar to that observed in earlier model studies.[9] The alkynes are bent substantially from linearity while the alkene is essentially normal. The angles for the two different conformations are as follows: C24, 161.9° and 169.6°; C23, 165.5° and 164.0°; C27, 160.2° and 172.5°; and C28, 168.3° and 162.1°. Since the estimated standard deviation in these measurements is ±2°, it is not clear that the two different crystal conformations observed for the anthraquinone portion have structural consequences for the 1,5-diyn-3-ene fragment. The methyl group at C4, $C29H_3$, makes a close approach to the 1,5-diyn-3-ene bridge with a closest distance of 2.93 (3) Å and 3.07 (3) Å to C23. Carbons 23 and 28 are separated by 3.54 (3) Å.[9]

With the structures of 1 and 2 fully defined, the structures of some of the minor dynemicins–L (3, $C_{30}H_{22}NO_9Cl$), M (4, $C_{29}H_{23}NO_9$), and N (5, $C_{30}H_{23}NO_{10}$)—could be established spectroscopically.[10] The ^{13}C NMR spectra of 3–5 exhibited signals for the anthraquinone moiety essentially identical with those of 1. The spectrum of 3 lacked both the characteristic four singlet carbon resonances of the conjugated acetylene (δ_C 88.8–99.4) and the two doublet protons of the alkene (δ_H 6.06 and 6.09) observed in 1 and 2. The presence of four new sp^2 carbon signals around δ_C 126.2–135.6 and signals for four contiguous aromatic protons (δ_H 7.10–7.60) strongly argued that the 1,5-diyn-3-ene had aromatized. The epoxide had also disappeared and been replaced by a chlorine at C8 and a hydroxyl at C3 as indicated by homo- and heteronuclear shift correlation spectroscopies. Thus dynemicin L is 3. Dynemicin N (5) was very similar to 3, but its molecular formula and spectroscopic data indicated that the chlorine in 3 was replaced by a hydroxyl in 5. The ^{13}C NMR spectrum of dynemicin M (4) was generally similar to the spectra of 3 and 5, but displayed a new ketone (δ_C 204.8) and a new sp^3 carbon (δ_C 82.7, d) at the expense of the carboxyl (δ_C 167) and the two sp^2 carbons (δ_C 113–114 and 153–159). Structure 4 is fully consistent with the spectral data for dynemicin M. Further support for structures 3 and 5 was provided by allowing 1 to stand in an acidic solution at room temperature and watching its rapid conversion to 3 followed by the gradual appearance of 5. This decomposition experiment also indicates that the epoxide ring can open easily to relax the strain of the 1,5-diyn-3-ene embedded in the 10-membered ring and bring C23 and C28 close enough to cyclize to an aromatic ring. This mechanism, with the replacement of the epoxide by a bridgehead double bond, is the proposed mode of action of the esperamicin/calicheamicin class of antibiotics.[4] The epoxide of dynemicin A (1) is also reminiscent of the epoxide in the neocarzinostatin chromophore, but in the neocarzinostatin case, opening of the epoxide yields an isolable chlorohydrin.[11] The decomposition experiment also raises the possibility that dynemicins L (3), N (5), and possibly M (4) are artifacts of the isolation.

Dynemicin A (1) and triacetyldynemicin A (2) display strong activity against Gram-positive bacteria with 2 being 2–8 times more active against a range of test organisms. Both compounds show marked cytotoxic activity against B16 melanoma, Moser human carcinoma, HCT-116 human carcinoma, and the normal and vincristine-resistant P388 leukemia cells with IC_{50} values of 0.004–0.005 μg/mL. In in vivo tests, both 1 and 2 produced significant prolongation of life span in P388 leukemia and B16 melanoma inoculated mice. Unlike the esperamicin antibiotics, 1 exhibits significant in vivo antibacterial activity and low toxicity. Dynemicins L, M, and N do not show significant levels of activity.

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Supplementary Material Available: ^1H NMR spectral data for triacetyldynemicin A (2), physicochemical properties and spectral data for dynemicins L (3), M (5), and N (4), and X-ray crystallographic data for triacetyldynemicin A (2) (21 pages). Ordering information is given on any current masthead page.

(8) Triacetyldynemicin A (2): orange rods from aqueous MeOH; mp 228–231 °C dec; $[\alpha]_D$ +1300 (*c* 0.05, MeOH) λ_{max} 224 (ε 40 100), 313 (6700), and 482 nm (8100); ^1H NMR (DMSO-d_6, 400 MHz) δ 1.25 (3 H, d, J = 7.3, 29-H), 2.33, 2.36, and 2.44 (3 H each, s, $COCH_3$), 3.55 (1 H, q, J = 7.3, 4-H), 3.79 (3 H, s, 31-H), 4.78 (1 H, s, 7-H), 5.04 (1 H, d, J = 3.8, 2-H), 6.05 (1 H, dd, J = 9.8 and 1.3, 25-H), 6.07 (1 H, dd, J = 9.8 and 1.3 Hz, 26-H), 7.62 (2 H, s, 16-H and 17-H), 8.03 (1 H, s, 10-H), 9.41 (1 H, d, J = 3.8, 1-NH), 12.37 (1 H, br, 30-OH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 43.8 (d, C2), 71.3 (s, C3), 35.6 (d, C4), 114.8 (s, C5), 153.2 (s, C6), 31.4 (d, C7), 63.0 (s, C8), 130.1* (s, C9), 130.0* (d, C10), 139.5 (s, C11), 124.5 (s, C12), 80.6 (s, C13), 125.9 (s, C14), 146.4 (s, C15), 131.0 (d, C16), 130.6 (d, C17), 146.9 (s, C18), 126.1 (s, C19), 182.7 (s, C20), 114.7 (s, C21), 143.8 (s, C22), 97.3 (s, C23), 89.6 (s, C24), 124.4 (d, C25), 124.0 (d, C26), 88.8 (s, C27), 99.4 (s, C28), 18.5 (q, C29), 167.3 (s, C30), 57.7 (s, C31), 20.6 × 2 and 20.9 ($COCH_3$), 168.9 and 169.1 × 2 ($COCH_3$). Assignments with an asterisk (*) may be interchanged. Anal. Calcd for $C_{34}H_{25}NO_{17}\cdot H_2O$: C, 63.43; H, 3.99; N, 2.06. Found: C, 63.20; H, 3.75; N, 2.16.

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(10) 3: blue powder; mp 222–225 °C; $[\alpha]_D$ −820° (*c* 0.10, MeOH); λ_{max} 241 (ε 48 100), 454 (2400), 594 (18 000), 639 nm (17 900). 4: blue powder; mp 238–240 °C; $[\alpha]_D$ −2460° (*c* 0.01, MeOH); λ_{max} 241 (ε 41 700), 453 (1500), 589 (17 000), 633 nm (17 200). 5: blue powder; mp 253–256 °C; $[\alpha]_D$ −200° (*c*, 0.01, MeOH); λ_{max} 241 (53 100), 452 (4300), 592 (24 500), 639 nm (25 200).

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EXHIBIT I

Communication to the Editor

DYNEMICIN A, A NOVEL ANTIBIOTIC WITH THE ANTHRAQUINONE AND 1,5-DIYN-3-ENE SUBUNIT

Sir:

The 1,5-diyn-3-ene-containing antibiotics represented by esperamicin[1,2] and calicheamicin[3] are receiving increasing attention because of their extremely potent antitumor activity and unusual structures. A unique mechanism of action involving phenyl diradical formation has been proposed for this family of antibiotics[4]. These antibiotics show extremely strong inhibition of growth of Gram-positive bacteria, especially the recombination-deficient mutants such as *Bacillus subtilis* M45 strain. During the course of our continuing search for new antitumor antibiotics using *B. subtilis* M45, dynemicin A, a novel violet-colored antibiotic was discovered in the fermentation broth of a new *Micromonospora* strain. The antibiotic exhibits very potent antibacterial activity, especially against Gram-positive bacteria, and prolongs the life span of mice inoculated with P388 leukemia. Structural studies revealed that dynemicin A is a unique hybrid of an anthraquinone and an 1,5-diyn-3-ene system. This communication describes the production, isolation, physicochemical properties, structure, and biological activities of dynemicin A.

The producing organism was isolated from a soil sample collected in Gujarat State, India and was identified as *Micromonospora chersina* sp. nov. No. M956-1. Antibiotic production was carried out in two 200-liter tank fermenters containing 120 liters each of a production medium (soluble starch 1.5%, glucose 0.5%, beet molasses 1%, fish meal 1% and $CaCO_3$ 0.5%, pH 7.0 before sterilization) at 28°C with agitation (250 rpm) and aeration (120 liters/minute). The antibiotic activity reached a maximum at 92 hours, as monitored by the paper-disc assay using *B. subtilis* PCI 219 as the test organism.

Dynemicin A (**1**) was isolated from the cultured broth by the following procedure. The whole broth (220 liters) was adjusted to pH 2.0 with 6 N HCl and extracted with BuOH (80 liters). The extract was concentrated *in vacuo* to an aqueous solution (1 liter) which deposited a dark brown precipitate. The solid collected by filtration was dissolved in MeOH (2 liters), combined with the aqueous filtrate and then loaded onto a column of Diaion HP-20 (10 i.d.×65 cm) previously equilibrated with 70% aqueous MeOH. After being washed with 80% MeOH, the activity was eluted from the column with 80% aqueous acetone. The residue (62 g) obtained upon concentration of the active eluate was rechromatogaphed on a column of Sephadex LH-20 (4 i.d.×40 cm) with MeOH elution.

Table 1. Physico-chemical properties of dynemicin A and triacetyldynemicin A.

	Dynemicin A	Triacetyldynemicin A
Nature	Violet amorphous powder	Orange rods
MP (°C, dec)	208~210	228~231
$[\alpha]_D^{24}$	+270° (*c* 0.01, DMF)	+1,300° (*c* 0.05, MeOH)
UV λ_{max}^{MeOH} nm (ε)	239 (24,900), 282 (sh), 569 (10,800), 599 (10,100)	244 (40,100), 313 (6,700), 482 (8,100)
Molecular formula	$C_{30}H_{19}NO_9$	$C_{36}H_{25}NO_{12}$
Microanalysis		Calcd for $C_{36}H_{25}NO_{12}\cdot H_2O$: C 63.43, H 3.99, N 2.06 / Found: C 63.20, H 3.75, N 2.16
SI-MS *m/z*, $(M+H)^+$	538	664
TLC[a] (Rf)	0.40	0.33

SI-MS: Secondary ion mass spectrum. [a] SiO_2; Xylene - methyl ethyl ketone - MeOH (5 : 5 : 1).

Fig. 1. IR spectrum of dynemicin A (KBr).

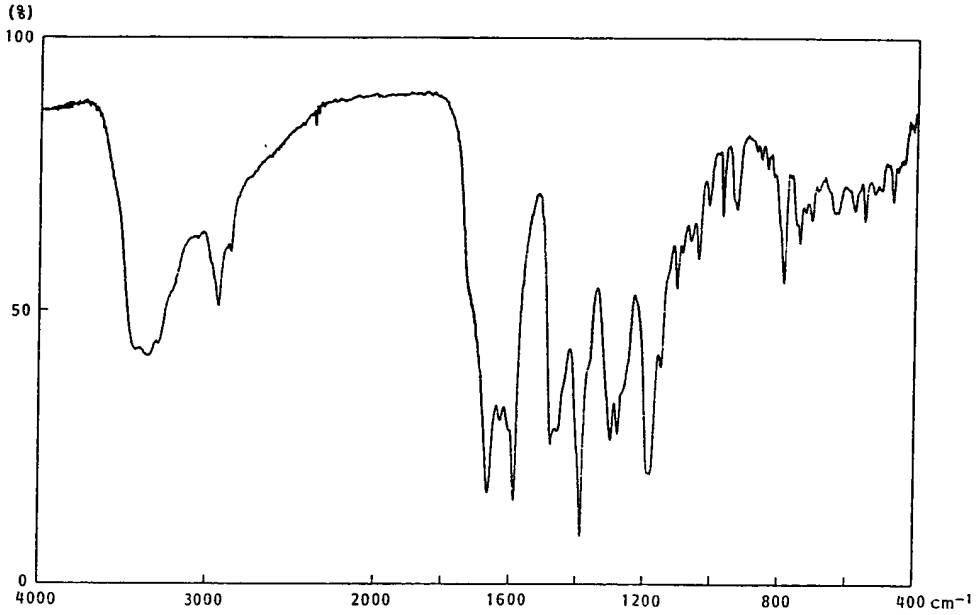


Table 2. ^{1}H NMR spectrum of triacetyldynemicin A (400 MHz in DMSO-d_6).

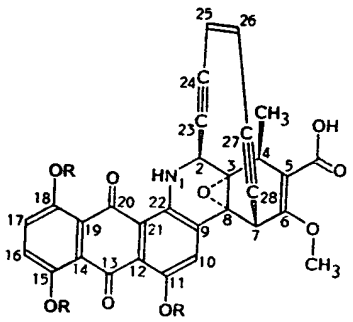
Proton No.	Triacetyldynemicin A
4-CH_3	1.25 (3H, d, J=7.3 Hz)
11-$OCOCH_3$, 15-$OCOCH_3$, 18-$OCOCH_3$	2.33 (3H, s), 2.36 (3H, s), 2.44 (3H, s)
4-H	3.55 (1H, q, J=7.3 Hz)
6-OCH_3	3.79 (3H, s)
7-H	4.78 (1H, s)
2-H	5.04 (1H, d, J=3.8 Hz)
25-H	6.05 (1H, d, J=1.3 Hz)
26-H	6.07 (1H, d, J=1.3 Hz)
16-H, 17-H	7.62 (2H, s, ×2)
10-H	8.03 (1H, s)
1-NH	9.41 (1H, d, J=3.8 Hz)
5-COOH	12.37 (1H, br)

Upon monitoring by TLC (see Table 1), the appropriate eluate was concentrated to yield a dark blue solid (56 mg). This was further purified by preparative TLC (SiO_2 and the same solvent as above TLC) followed by Sephadex LH-20 chromatography with MeOH elution to yield a homogeneous violet sample of **1** (5.7 mg).

1 is a violet amorphous solid soluble in DMSO, DMF and dioxane, slightly soluble in $CHCl_3$, EtOAc and MeOH and insoluble in H_2O and *n*-hexane. When treated with acetic anhydride in pyridine, **1** yielded a triacetyl derivative (**2**) with increased solubility. Their physico-chemical properties are summarized in Table 1. The IR spectrum of **1** (Fig. 1) contains a broad OH/NH absorption band at 3500~3200 cm^{-1} and carbonyl absorption bands at 1660 and 1630 cm^{-1}. The latter bands suggest a quinone group in the molecule. The IR spectrum of **2** exhibits a strong carbonyl band at 1770 cm^{-1} in addition to the bands observed for the spectrum of **1**. One methyl (δ 1.25), three acetyl methyls (2.33, 2.36 and 2.44), one OCH_3 (3.79), three methines (3.55, 4.78 and 5.04), two olefinic (6.05 and 6.07) and three aromatic protons (7.62×2 and 8.03) were observed in the ^{1}H NMR spectrum of **2** (Table 2). Corresponding carbon signals were found in the

^{13}C NMR. The ^{1}H NMR spectrum and the UV spectrum of **1** resemble those of ε-isorhodomycinone[5,6] suggesting a 1,4,6,9-tetrahydroxyanthraquinone or a related chromophore in the molecule. The remaining part of the molecule should have one CH_3, one OCH_3, three >CH, two –CH=, two >C=, six quaternary carbons and one carboxyl carbon. Among the quaternary carbons, four carbons appeared at δ 88.8, 89.6, 97.3 and 99.4 strongly suggesting a conjugated diyne system from spectral comparison with esperamicin. The complete structure was elucidated by X-ray crystallography of crystalline **2**[†] (Fig. 2).

Fig. 2. The structures of dynemicin A and triacetyldynemicin A.



Dynemicin A R=H
Triacetyldynemicin A R=$COCH_3$

Dynemicin A (**1**) and its triacetate (**2**) showed extremely strong activity against Gram-positive bacteria as shown in Table 3. Gram-negative bacteria, anaerobic bacteria and fungi are considerably less sensitive to both compounds. On the whole, **2** is two to eight times more potent than **1** against the organisms tested. **1** exhibited significant *in vivo* activity against *Staphylococcus aureus* Smith infection in mice with the PD_{50} being 0.13 mg/kg by ip administration. No toxic signs were observed in the mice after administration of 5 mg/kg (ip) of **1**. Both compounds showed marked cytotoxic activity against B16 melanoma, Moser human carcinoma, HCT-116 human carcinoma and the normal and vincristin-resistant P388 leukemia cells with IC_{50} of 0.004~0.005 μg/ml. In *in vivo* tests, **1** and **2** produced significant prolongation of life span of mice inoculated with P388 leukemia and B16 melanoma (Table 4). The active dose ranges were rather broad though the T/C values were maintained at not very high levels.

It is apparent that dynemicin A is a new member of the esperamicin/calicheamicin family of antibiotics in terms of possessing the unique 1,5-diyn-3-ene unit. However, it is distinctly different from the preceding antibiotics in possessing the violet-colored chromophore of a substituted anthraquinone. It should also be noted that unlike the esperamicin antibiotics, **1**

Table 3. Antibacterial spectra of dynemicin A and triacetyldynemicin A.

Organism	MIC (μg/ml)	
	Dynemicin A	Triacetyldynemicin A
Staphylococcus aureus FDA 209P	0.000013	0.0000063
S. aureus Smith	0.000025	0.0000063
S. epidermidis D153	0.0000063	0.0000031
Micrococcus luteus PCI 1001	0.0008	0.0002
Bacillus subtilis PCI 219	0.0000063	0.0000031
Escherichia coli NIHJ	0.05	0.0063
Klebsiella pneumoniae D11	0.0063	0.0008
Pseudomonas aeruginosa A9930	0.025	0.0063
Proteus vulgaris A9436	0.0063	0.0031
Clostridium difficile A21675	0.0063	0.0031
Bacteroides fragilis A22693	0.2	0.1
Candida albicans IAM 4888	12.5	0.4
Cryptococcus neoformans D49	12.5	0.8
Aspergillus fumigatus IAM 2530	6.3	0.1
Trichophyton mentagrophytes D155	12.5	0.4

† Full details of the structure determination will be forthcoming; M. KONISHI, H. OHKUMA, T. OKI, H. KAWAGUCHI and J. CLARDY.

Table 4. Antitumor activity of dynemicin A.

	P388 leukemia		B16 melanoma	
	Dose qd 1→3, ip (mg/kg/day)	T/C (%)	Dose qd 1→9, ip (mg/kg/day)	T/C (%)
Dynemicin A	1.0	135*	1.0	159*
	0.5	130	0.5	137
	0.25	135	0.25	137
	0.13	130	0.13	122
	0.063	130	0.063	141
	0.031	130	0.031	122
Mitomycin C	3.0	200	2.0	222
	1.0	135	1.0	152
	0.3	140	0.5	133
	0.1	115	0.25	111

* T/C≧125 means significant antitumor effect.

exhibited significant *in vivo* antibacterial activity and low toxicity. The anthraquinone chromophore moiety of dynemicin A is presumed to play an important role in its biological activity and further studies on the subject will be pursued.

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EXHIBIT J

and *Claviceps* spp., respectively. In addition, we have found that field-produced sclerotia formed by inoculation of corn ears in field test plots contain quantities of nominine similar to those found in laboratory-produced sclerotia. Taken together, these results suggest a possible ecological role for sclerotial metabolites and provide further evidence that fungal sclerotia are a unique and promising source of new bioactive natural products.

Experimental Section

General Procedures. Sclerotia from a strain of *A. nomius* (NRRL 13137) were obtained from the USDA Northern Regional Research Center in Peoria, IL. The sclerotia were prepared by solid substrate fermentation on autoclaved corn kernels using general procedures which have been previously described[3] and were stored at 4 °C until extraction. Proton and carbon NMR data were obtained in $CDCl_3$ on a Bruker WM-360 spectrometer, and chemical shifts were recorded using the signal for the residual protiated solvent (7.24 ppm) as a reference. Carbon multiplicities were established by a DEPT experiment. One-bond C–H correlations were obtained using an XHCORR pulse sequence optimized for 135 Hz. Proton signals studied with the selective INEPT technique were individually subjected to three separate experiments, optimizing for 7, 10, or 13 Hz. HREIMS data were obtained on a VG ZAB-HF instrument. Details of other experimental procedures and insect bioassays have been described elsewhere.[13,14]

Isolation and Properties of Nominine (2). Sclerotia of *A. nomius* (500–750 μm diameter, 53 g) were ground with a mortar and pestle and triturated repeatedly with hexane (5 × 200 mL). The combined hexane extracts were filtered and evaporated to afford 117 mg of a light yellow oil. This residue was subjected to reversed-phase semipreparative HPLC (5μ C_{18} column; 250 × 10 mm; 90:10 MeOH–H_2O at 2.0 mL/min) to afford 23.7 mg of nominine (2) as an off-white powder. The retention time for 2 under these conditions was 22.2 min. Compound 1: mp 54–55 °C; $[\alpha]_D$ +23.6° (*c* 0.85, MeOH); ^1H NMR and ^{13}C NMR ($CDCl_3$), Table I; EIMS (70 eV) 405 (M^+; rel intensity 57), 387 (100), 318 (15), 304 (52), 302 (70), 288 (17), 248 (15), 232 (15), 196 (42), 180 (40), 168 (28), 156 (16); HREIMS obsd 405.3035, calcd for $C_{28}H_{39}NO$ 405.3031.

Liquid Culture of *A. nomius*. A sterilized medium suitable for production of sclerotia on agar in petri dishes (1.5% glucose and 0.5% yeast extract; 50 mL) was inoculated with *A. nomius* and aerated by agitation on an orbital shaker at 200 rpm for 28 days. Although the fungus produced substantial mycelial growth under these conditions, sclerotia were not formed, and no trace of nominine was detected in organic extracts of the mycelium or the culture filtrate by analytical HPLC.

Detection of Nominine in Sclerotia from Field-Inoculated Corn. Several silking corn ears on growing corn plants at the USDA Northern Regional Research Center field plot were toothpick-wound inoculated with a conidial suspension of *A. nomius*. When the ears reached full maturity, small quantities of sclerotia were harvested from the ears and manually separated from all other fungal and plant material. Extraction of these sclerotia with $CHCl_3$ and analysis of the extract by analytical HPLC under the conditions above indicated the presence of nominine at a level similar to that found in laboratory-produced sclerotia.

Acknowledgment. This work was conducted under Cooperative Research Agreement No. 58-5114-M-010 between the USDA Agricultural Research Service and the University of Iowa. We thank Dr. George Crull for helpful NMR discussions.

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The Structures of Pradimicins A, B, and C: A Novel Family of Antifungal Antibiotics

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The structures of the novel antifungal antibiotics pradimicins A, B, and C, elaborated by a new strain of *Actinomadura hibisca*, have been determined on the basis of chemical degradations and spectral analysis. Acid hydrolysis cleaved pradimicin A to yield D-xylose, 4,6-dideoxy-4-(methylamino)-D-galactose, an aromatic chromophore fragment, and D-alanine. Extensive homo- and heteronuclear 2D NMR experiments assisted by the degradation results allowed us to assign *N*-[[(5*S*,6*S*)-5-*O*-[4,6-dideoxy-4-(methylamino)-3-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl]-5,6,8,13-tetrahydro-1,6,9,14-tetrahydroxy-11-methoxy-3-methyl-8,13-dioxobenzo[*a*]-naphthacen-2-yl]carbonyl]-D-alanine for the structure of pradimicin A. Pradimicins B and C are desxylosyl and des-*N*-methyl analogues of pradimicin A, respectively.

Introduction

Although enormous screening efforts have been made in the past 30 years, there are relatively few antifungal antibiotics with clinical efficacy, particularly against systemic fungal infections. In our efforts to discover microbial metabolites active against fungal infections, we have found that cultured broth of *Actinomadura hibisca* No. P157-2 (ATCC 53557), isolated from a soil sample from Fiji Island, contained red pigments that strongly protected mice from lethal infections caused by *Candida*, *Aspergillus*, and *Cryptococcus* strains.[1] The active principals were precipitated from the broth filtrate at pH 5.0 and purified by column chromatography to yield three components, pradimicins A (1a), B (1b), and C (1c).[2] In the in vitro assay,

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0022-3263/89/1954-2532$01.50/0

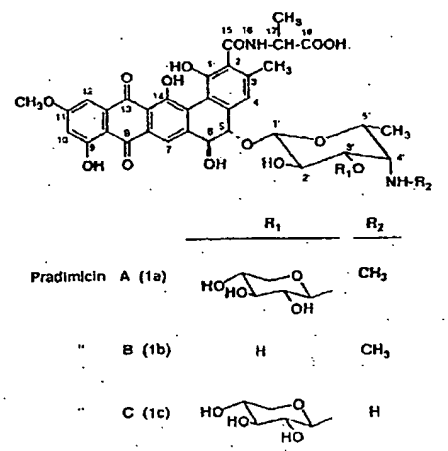

Figure 1. Structures of pradimicins A, B, and C.

these antibiotic components inhibited growth of a variety of fungi and yeasts with the minimum inhibitory concentration (MIC) ranging from 0.8 to 6.3 mcg/mL, while Gram-positive and Gram-negative bacteria were not sensitive. The three antibiotics also showed strong protective effects in mice experimentally infected with *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* strains. We report herein the structure elucidation of pradimicins A (**1a**), B (**1b**), and C (**1c**), which have a novel 5,6-dihydrobenzo[*a*]naphthacene chromophore substituted with D-alanine and sugars as shown in Figure 1.

Results and Discussion

The major component of the complex was **1a**, and it was isolated as an orange red amorphous powder of zwitterionic nature: mp 193–195 °C dec; $[\alpha]^{26}_D$ +685° (*c* 0.1, 0.1 N HCl); IR (KBr) 3400, 1605, and 1520 cm^{-1}; UV λ_{max} (50% MeOH) 231 nm (ϵ 28 300), 284 (22 700), and 482 (9600). Sodium salt of **1a** was obtained as dark red needles from a mixture of methyl acetate and 1-propanol. The molecular formula of $C_{40}H_{44}N_2O_{18}$ was established for **1a** based on the combustion analysis and high-resolution fast atom bombardment mass spectrometry (HR-FAB-MS, MH^+: obsd *m/z* 841.265 10, calcd 841.266 74).[3] **1b**: mp 195–198 °C dec; $[\alpha]^{26}_D$ +440° (*c* 0.1, 0.1 N HCl); $C_{35}H_{36}N_2O_{14}$; *m/z* 709 (MH^+). **1c**: mp 220–225 °C dec; $[\alpha]^{26}_D$ +619° (*c* 0.1, 0.1 N HCl); $C_{39}H_{42}N_2O_{18}$; *m/z* 827 (MH^+). The UV and IR spectra of **1b** and **1c** are very similar to those of **1a**, demonstrating that they share a common chromophore. The ^{13}C NMR spectrum of **1a** displayed 40 signals composed of CH_3-C × 3, CH_3-N × 1, CH_3-O × 1, CH_2-O × 1, CH-N(or O) × 12, CH= × 4, >C= × 14, amide or carboxylic acid × 2 (δ 168.9 and 174.6) and quinone carbonyl × 2 (δ 180.6 and 187.5). Acid methanolysis (1.5 N HCl–MeOH, reflux, 4 h) cleaved **1a** to a chromophore fragment (**2**), mp 245–248 °C dec, $C_{36}H_{38}N_2O_{14}$, *m/z* 723 (MH^+), and

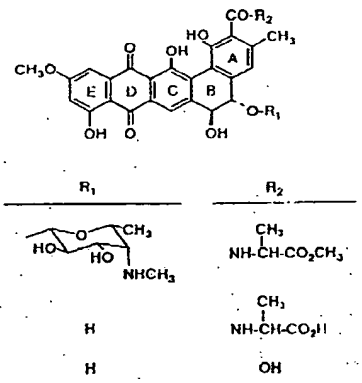

Figure 2. Structures of degradation products **2**, **3**, and **5**.

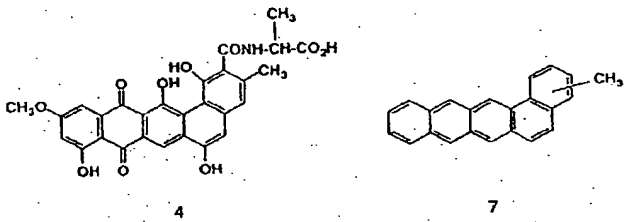

Figure 3. Structures of degradation products **4** and **7**.

methyl D-xyloside. The NMR spectrum of **2** contained an OCH_3 group (δ_H 3.68, s, and δ_c 51.4) which had not been observed in **1a**. Upon heating with 0.1 N NaOH for 1 h, **2** split off the OCH_3 group yielding **1b**, the bioactive minor component of pradimicin, which conversely converted to **2** by acid methanolysis. Vigorous acid hydrolysis of **1a** or **2** (6 N HCl, 115 °C, 14 h) afforded a major aglycon **3** (mp 221–223 °C dec; $[\alpha]^{26}_D$ −140° (*c* 0.1, MeOH), $C_{28}H_{23}NO_{11}$; *m/z* 550 (MH^+)), two minor aglycones (**4** and **5**) ($C_{28}H_{21}NO_{10}$, *m/z* 532 (MH^+), mp >250 °C, $C_{25}H_{18}O_{10}$, *m/z* 479 (MH^+), mp 207–210 °C dec), and D-alanine.[4,5] The presence of an amino sugar in **1b** had been postulated by the calculated molecular formula difference between **1b** and **3**. Although it could not be isolated from the above hydrolyzate possibly due to decomposition during hydrolysis, it was obtained as a mixture of α and β anomers (78:22, **6a** and **6b**) by acid methanolysis of *N*-acetylpradimicin A. The ^1H–^1H 2D NMR experiments and optical rotational value allowed us to assign the methyl 4,6-dideoxy-4-(methylamino)-D-galactopyranoside structure to the sugar. Compound **3** retained the UV spectrum and, in the ^{13}C NMR, all 22 sp^2 carbons (18 aromatic and 4 carbonyl carbons), two C-CH_3, one O-CH_3, and three methines observed for the parent antibiotic. Upon distillation with zinc dust, **3** afforded a methylbenzo[*a*]naphthacene (**7**), $C_{23}H_{16}$, *m/z* 292 (M^+), UV λ_{max} 220, 252, 258, 292, 302, 316, 355, 374, 398, 422, and 449 nm, demonstrating a benzo[*a*]naphthacene nucleus to the antibiotic. The ^{13}C signals of **3** (also of **1a**) closely resembled some of those of 10-dihydrosteffimycin A[6] (rings D and E in

(2) While this publication was being written, the production and structure of new antifungal antibiotic benanomicins were published. Although stereochemistry of C5 and C6 of benanomicins has not been reported, one of the components, benanomicin B, was identified with pradimicin C by a direct comparison. Takeuchi, T.; Hara, T.; Naganawa, H.; Okada, M.; Hamada, M.; Umezawa, H.; Gomi, S.; Sezaki, M.; Kondo, S. *J. Antibiot.* **1988**, *41*(6), 807. Gomi, S.; Sezaki, M.; Kondo, S.; Hara, T.; Naganawa, H.; Takeuchi, T. *J. Antibiot.* **1988**, *41*(8), 1019.

(3) We observed abundant $(M + 3H)^+$ ions of pramidicins A, B, and C and their hydroysis products ions in FAB-MS and secondary-ion MS. This was due to reduction of the quinone of these compounds to the hydroquinone in the mass spectrometer which adsorbed one proton. Misra, R.; Pandy, R. C.; Silverton, J. V. *J. Am. Chem. Soc.* **1982**, *104*, 4478. Cooper, R.; Unger, S. *J. Antibiot.* **1985**, *38*(1), 24.

(4) The D configuration was assigned based on the HPLC behavior on a chiral HPLC column (MIC Gel ODS-1HU, Mitsubishi Kasei). Elution: 2 mM *N,N*-dipropyl-L-alanine and 1 mM copper acetate solution, pH 5.7.

(5) In the acid hydrolysis, major aglycon **3** precipitated rapidly (85% yield), and it was resistant to further hydrolysis. HPLC analysis of the hydrolyzate indicated the presence of aglycone **5** and D-alanine in a comparable ratio (ca. 5% yield). The peptide bond was found to be unusually stable to acid and alkaline hydrolysis (6 N HCl and 6 N NaOH, 110 °C). The ^1H and ^{13}C NMR data evidenced the presence of 1 M D-alanine in this molecule.

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Table I. ^{13}C NMR Data of 1a, 1b, 1c, 2, and 3 (100 MHz, DMSO-d_6)

carbon	1a	1b	1c	2	3
1	157.6	157.3	157.5	157.9	157.1
2	126.9	126.6	126.9	125.7	125.9
3	136.5	136.3	136.3	136.8	136.2
4	116.9	116.7	116.8	116.6	114.9
4a	137.7	137.6	137.5	137.5	140.5
5	82.7	82.1	82.5	82.0	71.6
6	71.9	71.8	71.9	71.6	72.4
6a	143.7	143.6	143.6	143.6	145.3
7	111.6	111.0	111.2	111.2	110.7
7a	132.2	131.9	132.0	131.7	131.8
8	187.5	187.3	187.2	187.1	187.2
8a	110.5	110.2	110.3	110.2	110.2
9	164.1	163.9	163.9	163.8	163.8
10	104.4	104.0	104.3	104.1	103.9
11	166.0	165.7	165.8	165.6	165.5
12	106.3	105.9	106.0	105.7	105.7
12a	138.0	137.9	137.8	137.6	137.9
13	180.5	180.2	180.3	180.2	180.2
13a	119.3	119.0	119.0	118.7	118.6
14	166.4	166.7	166.5	165.4	166.8
14a	133.1	133.2	133.0	132.4	133.3
14b	119.0	118.8	118.8	118.5	118.5
15	168.9	168.2	168.2	168.1	168.1
17	48.2	47.7	48.0	47.5	47.8
18	174.6	174.3	174.5	173.0	174.2
3-CH_3	20.0	20.0	19.9	20.0	19.8
11-OCH_3	56.2	55.9	56.0	55.8	55.7
17-CH_3	17.6	17.3	17.5	17.0	17.4
18-$COOCH_3$				51.4	
1′	104.5	104.8	104.4	104.6	
2′	70.2	71.2	69.8	71.0	
3′	80.4	71.0	79.8	70.6	
4′	63.4	64.0	54.3	63.9	
5′	67.9	67.9	67.6	67.5	
5′-CH_3	16.4	16.3	16.4	16.0	
4′-NCH_3	36.6	36.8		36.5	
1″	105.3		105.1		
2″	73.8		73.5		
3″	76.1		76.0		
4″	69.6		69.4		
5″	66.0		65.8		

Figure 2, C8–C13 in Table I) and those of cosmocarcin A[7] (rings C and D, C6a–C8a and C12a–C14a). The ^1H NMR data also supported the similarity to those antibiotics indicating that 3 possesses a 2-methoxy-4,9-dihydroanthraquinone partial structure. The extensive homo- and heteronuclear two-dimensional NMR studies including long-range ^1H–^{13}C COSY and NOESY revealed the structure of the remaining part of the core moiety. The experiments were conducted with 2, which was the most soluble of the pradimicin analogues in the NMR solvent (DMSO-d_6). Analysis of the data allowed the unambiguous assignment of all the carbon (Table I) and proton signals of 2. In the long-range ^1H–^{13}C COSY, C3-methyl protons displayed cross peaks with C2, C3, and C4; H4 with C2, C14b, and the methyl carbon attached to C3; H5 with C4a, C6, C6a, C14b, and C1′ (anomeric carbon of 6); and H6 with C4a, C6a, and C7, establishing the substitution pattern of rings A, B, and C. (Figure 4). The spectrum also substantiated the assigned structure of rings C, D, and E and the D-alanine bonded to C2-carboxylic acid. The NOESY spectrum of 2 solidly supported this structure (Figure 4). In the ^1H NMR spectrum of 1a and 2, H5 coupled to H6 with J value of ca. 10.0 Hz, indicating that they were in a trans-diaxial orientation. A negative first ($\Delta\epsilon$ = −30.6 at 225.4 nm) and positive second Cotton effect ($\Delta\epsilon$ = +16.14 at 205.8 nm) observed for 3 indicated a

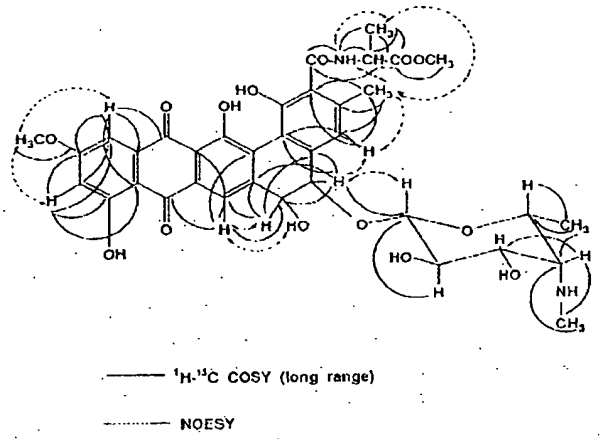

Figure 4. Long-range ^1H–^{13}C COSY and NOESY spectra of 2.

negative helicity of the axis between the plane of ring A and rings C, D, and E.[8,9] These results proved a 5*S*,6*S* absolute configuration of ring B of the antibiotic. The β-pyranoside linkage of the sugar to C5-OH was assigned by the magnitude of the coupling of the anomeric proton (H1′, δ 4.63, d, J = 8.1 Hz). The molecular formula of 4, $C_{28}H_{21}NO_{10}$, indicated that 4 was a dehydration product of 3. A pronounced bathochromic shift in its UV spectrum suggested that the dehydration extended the conjugation of the chromophore. The ^1H NMR spectrum of 4 showed an aromatic proton (δ 7.20) in addition to the four aromatic protons observed for 3. Instead, the two low-field methines assigned to H5 and H6 in 3 were missing in the spectrum of 4, verifying that the dehydration took place at C5-OH and C6-H. The UV absorption of 5 appeared at slightly longer wavelengths (λ_{max} 224, 274, 320, and 509 nm in alkaline solution) than those of 1a, 2, and 3. The NMR demonstrated that 5 possessed an identical aglycon structure with 3 but lacked the signals assignable to D-alanine of 3.

The ^1H and ^{13}C NMR of 1a assisted by two-dimensional NMR experiments evidenced a β-pyranoside configuration of the D-xylose (H1″, δ 4.44, d, J = 7.3 Hz). Previous mild hydrolysis experiment has identified 1b as desxylosyl-1a. In comparative ^{13}C NMR analysis, C3′ of 1a appeared 8.9 ppm lower field than that of 1b showing that the xylose was linked to C3′-OH in 1a.

The ^1H and ^{13}C NMR spectra of 1c correspond well with those of 1a, differing only in that the N-CH_3 (δ_H 2.61 and δ_C 36.6) in 1a was missing and the C4′ signal shifted upfield by 9.1 ppm in 1c. *N*-Desmethyl structure for 1c was confirmed by the isolation of methyl 4,6-dideoxy-4-amino-D-galactoside[10] in acid methanolysis of *N*-acetyl 1c. It was concluded from the results of the above investigation that pradimicins A (1a), B (1b), and C (1c) have the structures as illustrated in Figure 1, the degradation products 2, 3 and 5 as in Figure 2, and 4 and 7 as in Figure 3.

Experimental Section

Thin-layer chromatography (TLC) was performed on precoated silica gel plates (Kieselgel 60F_{254}, Merck, 0.25 mm thick). The IR spectra were determined on a JASCO IR-810 spectrometer

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and the UV spectra on a JASCO UVIDEC-610C spectrometer. The ^1H and ^{13}C NMR spectra were recorded on a JEOL JMN-GX 400 spectrometer operated in the Fourier transform mode using tetramethylsilane and/or dioxane as the internal standard. Electron-impact and secondary-ion mass spectra (EI-MS and SI-MS) were obtained with a Hitachi M80B mass spectrometer modified with an im-beam and SI-MS inlet. Fast atom bombardment mass spectra (FAB-MS) were run with a JMS-DX 303 HF spectrometer (JEOL) and high-resolution fast atom bombardment mass spectra (HR-FAB-MS) on a VG70SE spectrometer. Optical rotation were determined with a JASCO Model DIP-140.

Isolation of Pradimicins A (1a), B (1b), and C (1c). The fermentation broth (108 L) was centrifuged, and the supernatant was adjusted to pH 2.0 by 6 N HCl. After the brown precipitate was removed by filtration, the filtrate was neutralized to deposit a dark red crude solid of pradimicin. This solid was thoroughly stirred with a mixture of *n*-BuOH–MeOH–1% NaCl, pH 2.0 (3:1:4, 73 L). The organic layer was separated and extracted with aqueous NaOH (pH 8.0, 30 L). The aqueous layer was adjusted to pH 2.0 by 1 N HCl and subjected to column chromatography on Diaion HP-20 eluted with 60% aqueous acetone (pH 3.0). The red, active eluate was concentrated in vacuo to afford a semipure solid of the antibiotic (62 g). Three grams of the solid was subjected to reversed-phase silica gel chromatography (ODS-60, Yamamura Chemical Lab., 8 × 100 cm) developed with CH_3CN–0.15% KH_2PO_4, pH 3.5 (22:78, v/v). The major red eluate containing **1a** was concentrated in vacuo to an aqueous solution, which was desalted by Diaion HP-20 chromatography with 60% acetone (pH 3.0) elution, yielding a red homogeneous solid of **1a**·HCl (1.52 g). In this reversed-phase chromatography, the eluates before and after **1a** were pooled and further purified by reversed-phase silica gel and Diaion HP-20 chromatography to afford pure **1b**·HCl (140 mg) and **1c**·HCl (98 mg), respectively. Crystallization of **1a**·HCl from MeOAc–*n*-PrOH–0.1 N NaOH gave fine needles of the monosodium salt. The salt dissolved in water and adjusted to pH 5.0 by 0.1 N HCl, to yield a precipitate of the zwitterionic form of **1a**. Aqueous solutions of **1b**·HCl and **1c**·HCl were adjusted to pH 5.0 by 0.1 N NaOH to precipitate the zwitterionic form of **1b** and **1c**, respectively.

1a: mp 193–195 °C dec; $[\alpha]^{28}_D$ +685° (*c* 0.1, 0.1 N HCl); UV (50% MeOH) λ_{max} 231 nm (ε 28 300), 284 (22 700), 482 (9600); (0.01 N HCl–50% MeOH) λ_{max} 234 (31 100), 299 (26 600), 459 (11 100); (0.01 N NaOH–50% MeOH) λ_{max} 240 (33 300), 318 (14 700), 500 (15 100); IR (KBr) 3400, 1605, 1450, 1390, 1295, 1260, 1160, 1050 cm^{-1}; ^{13}C NMR (Table I); HR-FAB-MS (glycerol) *m/z* 841.2651 (MH^+); SI-MS (glycerol) *m/z* 843 $(M + 3H)^+$, (mNBA) *m/z* 841 (MH^+). Anal. Calcd for $C_{40}H_{44}N_2O_{18}·H_2O$: C, 55.94; H, 5.40; N, 3.26. Found: C, 55.99; H, 5.59; N, 3.24.

1b: mp 195–198 °C dec; $[\alpha]^{28}_D$ +440° (*c* 0.1, 0.1 N HCl); UV (MeOH) λ_{max} 234 nm (ε 30 100), 286 (24 100), 473 (10 100); (0.01 N HCl–MeOH) λ_{max} 234 (31 100), 296 (27 500), 460 (11 100); (0.01 N NaOH–MeOH) λ_{max} 241 (33 300), 316 (14 200), 504 (15 000); IR (KBr) 3380, 1610, 1450, 1390, 1295, 1260, 1165, 1130, 1070 cm^{-1}; ^{13}C NMR (Table I); SI-MS (glycerol) *m/z* 711 $(M + 3H)^+$; (mNBA) *m/z*, 709 (MH^+). Anal. Calcd for $C_{35}H_{36}N_2O_{14}·H_2O$: C, 57.85; H, 5.27; N, 3.85. Found: C, 57.79; H, 5.31; N, 3.85.

1c: mp 220–225 °C; $[\alpha]^{28}_D$ +619° (*c* 0.1, 0.1 N HCl); UV (50% MeOH) λ_{max} 230 nm (ε 31 400), 285 (23 400), 481 (9900); (0.01 N HCl–50% MeOH) λ_{max} 234 (32 700), 298 (27 800), 459 (11 500); (0.01 N NaOH–50% MeOH) λ_{max} 240 (32 200), 320 (14 900), 499 (15 400); IR (KBr) 3370, 1605, 1445, 1390, 1295, 1260, 1160, 1040 cm^{-1}; ^{13}C-NMR (Table I); SI-MS (mNBA) *m/z* 827 (MH^+).

Acid Methanolysis of 1a. A solution of **1a** (944 mg) in 1.5 N methanolic hydrogen chloride (60 mL) was refluxed for 4 h at 80 °C. The reaction mixture was diluted with water (200 mL) and extracted with *n*-butanol three times (100 mL each). The extract was concentrated in vacuo to give a crude solid of **2** (864 mg). A part of the solid (430 mg) was chromatographed on a column of reversed-phase silica gel (Lichroprep RP-18, 40-63 μm, Merck, 4 × 45 cm) developed with CH_3CN–0.15% KH_2PO_4 pH 3.5 (40:60, v/v). The eluate was collected in fractions which were examined by HPLC. (column: Microsorb Short One C18, 4.6 × 100 mm, 3 μm. Rainin Instrument Co. Elution: CH_3CN–0.15 KH_2PO_4, pH 3.5 (52:48). Flow rate: 1.2 mL/min. Detection: UV 254 nm. Retention time: **1a**, 2.03 min; **2**, 4.45 min.) The appropriate fractions were concentrated to an aqueous solution, which was charged on a column of Diaion HP-20 (200 mL) for desalting. The column was washed with water (500 mL) and eluted with 80% acetone (pH 3.0). Evaporation of the red effluent yielded pure **2** hydrochloride (144 mg). This salt was dissolved in water (15 mL) and adjusted to pH 5.0 with 0.1 N NaOH. The resultant precipitate was collected by filtration, washed with acetone, and dried in vacuo to give an amphoteric red powder of **2** (127 mg): mp 245–248 °C dec; $[\alpha]^{26}_D$ −340° (*c* 0.1, 0.1 N HCl); FAB-MS (glycerol) *m/z* 723 (MH^+); SI-MS (glycerol) *m/z* 725 $(M + 3H)^+$, (mNBA) *m/z* 723 (MH^+); UV (MeOH) λ_{max} 234 nm (ε 28 700), 283 (23 600), 476 (10 000); IR (KBr) 3400, 1735, 1625, 1440, 1385, 1255, 1160, 1070 cm^{-1}; ^{13}C NMR (Table I). Anal. Calcd for $C_{36}N_{38}N_2O_{14}·2H_2O$: C, 56.99; H, 5.58; N, 3.69. Found: C, 56.68; H, 5.05; N, 3.67.

The aqueous layer of the hydrolyzate was neutralized by Amberlite IR-45 resin (OH^-) and concentrated in vacuo to a small volume, which was loaded on a column of Diaion HP-20, which was developed with water. Evaporation of the anthrone-positive eluate yielded a pale-yellow solid (144 mg). This solid was chromatographed on a column of Sephadex G-10 eluted with H_2O, yielding methyl D-xyloside (131 mg): $[\alpha]^{28}_D$ +60° (*c* 2.0, H_2O); EI-MS *m/z* 164 (M^+). It was identical with an authentic sample in TLC and ^1H NMR spectrum.

Alkaline Hydrolysis of 2. A solution of **2** (230 mg) in 0.1 N NaOH (30 mL) was refluxed for 1 h. The reaction solution was acidified to pH 3.0 and applied to a column of Diaion HP-20 (250 mL). After washing with water, the column was eluted with 80% acetone (pH 3.0) to give pure **1b** hydrochloride (220 mg). This HCl salt was dissolved in water and adjusted to pH 5.0, yielding an amphoteric amorphous powder of **1b** (127 mg).

Acid Hydrolysis of 1a. A solution of **1a** (6.3 g) in 6 N HCl (275 mL) was heated at 115 °C for 14 h in a sealed container. After cooling the precipitate was collected by filtration. A part of the filtrate (1.2 mL) was passed through a column of Diaion HP-20 (20 mL). The ninhydrin-positive effluent was concentrated in vacuo to afford a white solid containing alanine (2.2 mg). The stereochemistry of the alanine was determined to be "D" by chiral HPLC. (Column: MCI Gel ODS 1HU, 4.6 × 150 mm, 5 μm, Mitsubishi Kasei. Elution: 2 mM *N,N*-dipropyl-L-alanine, 1 mM copper acetate, pH 5.7. Flow rate: 0.8 mL/min. Detection: UV 254 nm, t_R: D-Ala 4.8 min, L-Ala 6.6 min.) The precipitate obtained above was dissolved in aqueous NaOH (pH 11). The solution was adjusted to pH 6.0 by 1 N HCl, and the resultant precipitate was filtered off. The filtrate was loaded on a column of Diaion HP-20 (2.6 L). The column was washed with water and eluted with 60% acetone. Evaporation of the acetone eluate afforded 3.7 g of a red powder containing **3**, **4**, and **5**. This solid was purified by Sephadex LH-20 chromatography (3.6 L) with 50% MeOH as eluant. The first red eluate containing homogeneous **3** was concentrated in vacuo. **3** (3.2 g): mp 221–223 °C dec; $[\alpha]^{28}_D$ −140° (*c* 0.1, MeOH); FAB-MS (glycerol) *m/z* 550 (MH^+); SI-MS (glycerol) *m/z* 552 $(M + 3H)^+$; IR (KBr) 3370, 1600, 1445, 1390, 1295, 1255, 1160, 1130 cm^{-1}; UV (MeOH) λ_{max} 234 nm (ε 34 500), 287 (29 000), 478 (11 500); (0.01 N HCl–MeOH) λ_{max} 233 nm (35 300), 296 (29 900), 458 (12 600); (0.01 N NaOH–MeOH) λ_{max} 240 nm (37 400), 314 (14 800), 504 (14 600); ^{13}C NMR (Table I). Anal. Calcd for $C_{28}H_{23}NO_{11}·^1/_2H_2O$: C, 60.22; H, 4.33; N, 2.51. Found: C, 60.28; H, 4.40; N, 2.73.

Concentration of the second red eluate afforded **4** (20 mg): UV (50% MeOH) λ_{max} 237 nm (ε 22 000), 278 (24 400), 366 (11 500), 516 (9800); (0.01 N HCl–50% MeOH) λ_{max} 278 nm (14 800), 342 (7100), 477 (5000); (0.01 N NaOH–50% MeOH) λ_{max} 243 nm (22 600), 288 (29 400), 376 (10 500), 538 (9700); IR (KBr) 3400, 1615, 1480, 1440, 1365, 1290, 1240 cm^{-1}; FAB-MS (glycerol) *m/z* 532 (MH^+); molecular formula $C_{28}H_{21}NO_{10}$. The third red effluent from the Sephadex column was evaporated to dryness (87 mg), which was charged on a column of reversed-phase silica gel (ODS-60, Yamamura Chem. Lab. 22 × 450 mm). The column was developed with CH_3CN–0.03M phosphate buffer pH 7.0 (20:80), and the eluate was examined by HPLC. (Column: YMC A-301-3, Yamamura Chem. Lab. Elution: CH_3CN–0.03 M phosphate buffer pH 7.0 (25:75). Flow rate: 1.0 mL/min. Detection: UV 254 nm. t_R: 5.40 min.) The appropriate fractions were concentrated in vacuo and then desalted on a column of Diaion HP-20 to yield a homogeneous red powder of **5** (40 mg):

mp 207–210 °C dec; FAB-MS (glycerol) m/z 479 (MH^+); $C_{25}H_{18}O_{10}$; UV (50% MeOH) λ_{max} 237 nm (ϵ 29600), 283 (20100), 471 (9600); (0.01 N HCl–50% MeOH) precipitation; (0.01 N NaOH–50% MeOH) λ_{max} 224 (26900), 274 (23000), 320 (10400), 509 (11600); IR (KBr) 3400, 1600, 1440, 1380, 1290, 1255, 1185, 1160, 1120 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.58 (3 H, s), 3.89 (3 H, s), 4.17 (1 H, dd, J = 3.9 and 11.1), 4.26 (1 H, dd, J = 3.4 and 11.1), 5.69 (1 H, br s), 5.91 (1 H, br s), 6.67 (1 H, br d, J = 2.1), 6.86 (1 H, s), 7.05 (1 H, br d, J = 2.1), 8.02 (1 H, s), 13.05 (1 H, br s), 14.01 (1 H, s); ^{13}C NMR (DMSO-d_6) δ 186.3, 181.0, 170.1, 165.7, 165.7 164.5, 160.0, 145.9, 142.5, 137.5, 137.5, 132.1, 130.7, 125.9, 119.5, 117.8, 115.9, 113.2, 110.5, 105.9, 105.0, 72.2, 71.7, 55.9, 22.9.

Zinc Dust Distillation of 3. A mixture of 3 (50 mg) and zinc dust (500 mg) was placed in the bulb of a long glass tube (7 × 500 mm), which was then sealed. The bulb was heated over a burner to a red glow and kept for 30 s. After cooling, the tube was broken above the bulb and the upper piece containing the distillate was rinsed with diethyl ether. The ether extract was evaporated to dryness, which was developed on a preparative TLC plate (SiO_2, hexane–benzene, 9:1). The yellow band (R_f 0.41) was cut off and eluted from the silica gel with CH_2Cl_2. Evaporation of the solvent yielded a yellow liquid of 7: UV λ_{max} (n-heptane) nm 220, 252, 258, 292, 302, 316, 355, 374, 398, 422, 449; EI-MS m/z 292 (M^+). These data were consistent with a methyl benzo[a]naphthacene.

Isolation of Amino Sugar 6. Pradimicin A (600 mg) was treated with Ac_2O (6 mL) in MeOH (130 mL) at room temperature overnight. Concentration of the mixture in vacuo afforded a red solid of *N*-acetyl **1a** (558 mg). This solid (407 mg), without further purification, was hydrolyzed with 5.2 N HCl–MeOH (90 mL) under reflux temperature for 2.5 h. The reaction mixture was neutralized with 6 N NaOH and concentrated to an aqueous solution, which was loaded on a column of Diaion HP-20 (100 mL). The column was eluted with water, and the ninhydrin-positive eluate was evaporated. The residue was chromatographed on Amberlite CG-50 (H^+, 60 mL) with elution of 0.01 N HCl. The ninhydrin-positive fractions were pooled, concentrated to dryness (21.2 mg), charged on a column of Sephadex LH-20 (80 mL), and developed with 50% MeOH. Evaporation of eluate containing the sugar afforded a pale-yellow solid (6α and 6β, 7.5 mg): $[\alpha]^{26}_D$ +87.5° (c 0.3, H_2O); EI-MS m/z 191 (M^+), 160 ($M - OCH_3$)$^+$.

All protons of 6α and 6β was unequivocally assigned by the ^1H–^1H COSY experiment. Thus, **6** was identified as an anomeric mixture of methyl 4,6-dideoxy-4-(methylamino)-D-galactopyranoside. Pradimicin C was hydrolyzed in the same way as **1a** and yielded a pale-yellow solid of methyl 4,6-dideoxy-4-amino-D-galactoside mixture (6.2 mg, $\alpha:\beta$ = 78:22): $[\alpha]^{26}_D$ +89.8° (c 0.29, H_2O); SI-MS (glycerol) m/z 178 (MH^+), 200 ($M + Na$)$^+$.

Identity with methyl 4,6-dideoxy-4-amino-D-galactopyranoside was confirmed by a direct comparison with an authentic synthetic sample.[10]

Supplementary Material Available: ^1H NMR chemical shift data of **1a**, **1b**, **1c**, **2**, **3**, and **4** in dimethyl-d_6 sulfoxide and those of 6α, 6β, and methyl 4,6-dideoxy-4-amino-D-galactopyranoside in deuteroxide (3 pages). Ordering information is given on any current masthead page.

Biosynthesis of Pradimicin A

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The biosynthesis of pradimicin A (**1**) has been studied by feeding sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates and D- and L-[1-^{13}C]alanines to the producing organism *Actinomadura hibisca* sp. P157-2 (ATCC 53557). ^{13}C NMR spectroscopy established that the aglycon moiety of **1** is derived from 1 alanine unit and 12 acetate units, condensed in the "head-to-tail" fashion typical of polyketide biogenesis. Of particular interest is the efficient incorporation of D-alanine into **1**, suggesting that D-alanine might act as the direct precursor for the D-alanine side chain of **1**.

Introduction

Pradimicin A (**1**), a new antibiotic, has been found in the culture filtrate of *Actinomadura hibisca* sp. P157-2 (ATCC 53557).[1-3] The antibiotic is active in vitro against a wide variety of fungi and yeasts, some Gram-positive bacteria, and viruses. More interestingly, **1** demonstrates in vivo therapeutic activity against systemic fungal infections caused by *Candida albicans* A9540, *Aspergillus fumigatus* IAM2530, and *Cryptococcus neoformans* IAM4514 in mice. The closely related antibiotics benanomicins A (**2**) and B (**3**) have been reported to be produced by an actinomycete, MH193-16F4.[4] Structurally, all of these compounds contain a glycosylated benzo[a]naphthacenequinone that has a D-alanine side chain. As part of our microbial modification program, we initiated a biosynthetic study of pradimicin A by *A. hibisca* sp. P157-2. This paper presents the spectroscopic analysis of ^{13}C-labeled samples of **1**, which established the biosynthesis of the aglycon of **1**.

Results

[1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates and D- and L-[1-^{13}C]alanines were fed to cultures of *A. hibisca* sp. P157-2 to establish the biosynthetic origin of the aglycon moiety of **1**. The ^{13}C-enriched samples of **1** thus formed were isolated and the positions of the ^{13}C-enriched carbon atoms determined by ^{13}C NMR spectroscopy.

Acetate Connectivity in 1. Accurate chemical shift assignment of each carbon of **1** was essential in determining which pairs of carbons originate from the same molecule of acetate. In the initial ^{13}C NMR experiments chemical shifts of some of the carbons in **1** crossed over or coalesced at certain pH's, which seemed to occur due to the zwitterionic nature of **1**. However, when **1** was isolated as a water-insoluble solid by adjusting an aqueous solution of

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EXHIBIT K

Note

GLIDOBACTINS D, E, F, G AND H; MINOR COMPONENTS OF THE ANTITUMOR ANTIBIOTIC GLIDOBACTIN

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Glidobactin is a complex of novel antitumor antibiotics elaborated by *Polyangium brachysporum* sp. nov. No. K481-B101 (ATCC 53580). The three major components, glidobactins A, B and C have been isolated and their chemical and biological properties characterized[1]. They are unique acylpeptides structurally unrelated to any previously known antibiotics.

In our further investigation of the metabolites, five related congeners named glidobactins D, E, F, G and H were found as co-products of the major glidobactins A, B and C. Glidobactins D, E and F differ from glidobactin A only in their fatty acid side chains, while glidobactin G contains a hydroxymethyl group on the 12-membered core ring in place of the methyl group of the other glidobactins. The structure of glidobactin H has not yet been determined. This communication presents the isolation, properties, structures and biological activities of the new components.

The fermentation of *P. brachysporum* sp. nov. No. K481-B101 and isolation of the crude glidobactin solid were carried out as described before[1]. A portion of the solid (114 g) was loaded on a column of a reversed phase silica (Merck Kieselgel 60 silanised, 3.5 liters) which had been equilibrated with 70% aqueous methanol. Elution was carried out with 70% and 80% aqueous methanol and the eluates were pooled on the basis of the bioassay and HPLC (SSC-ODS-262, 80% aqueous methanol elution). Evaporation of the first pool yielded 16.3 g of semi-pure solid which contained glidobactins D, E, F, G and H. The work-up of the second and third pools afforded pure glidobactin A (14.6 g) and a mixture of glidobactins A, B and C (44.6 g), respectively.

The mixture of glidobactins D, E, F, G and H (12.7 g) was chromatographed on a column of silica gel (Wakogel C-200, 700 ml) developing with $CHCl_3$ - MeOH (10:1 and 5:1). The eluate was collected in fractions (18 ml each) which were analyzed by TLC (Merck Kieselgel $60F_{254}$, EtOH - H_2O, 55:45). The initial half of the active fractions (No. 91~160) was pooled, concentrated and lyophilized to afford 1.2 g of white solid which contained glidobactins F and H. The latter half was similarly worked up to give 930 mg of glidobactins D, E and G mixture. The glidobactins F and H complex (1.2 g) was chromatographed on reversed phase silica gel (160 ml) with MeOH - H_2O (1:1, 1 liter and 5:1, 2.5 liters). The first bioactive fractions were pooled, evaporated *in vacuo*, and lyophilized to give pure glidobactin F (131 mg). The second active fractions were evaporated to give a solid (88 mg) which was again purified by silica gel chromatography (column: 50 ml, elution: $CHCl_3$-MeOH) to isolate homogeneous glidobactin H (1.9 mg).

The complex of glidobactins D, E and G (930 mg) obtained above was again chromatographed on a silica gel column (Wakogel C-200, 190 ml). The elution was carried out by a mixture of $CHCl_3$ - MeOH (10:1, 1 liter and 5:1, 1.6 liters) and the active eluates were pooled and concentrated to give a purer solid of glidobactins D, E and G complex (97 mg). This complex was separated by reversed phase silica gel chromatography with aqueous methanol (50~70%) elution. The first active fractions were pooled and evaporated to give pure glidobactin D (8.4 mg). The second and the third active fractions were similarly worked up to isolate pure glidobactins E (5.1 mg) and G (18.1 mg), respectively.

The physico-chemical properties of glidobactins D, E, F, G and H are similar to those of the major components, glidobactins A, B and C. They were readily soluble in methanol, butanol and dimethyl sulfoxide, slightly soluble in chloroform, acetonitrile and ethyl acetate but insoluble in hexane and water. They were posi-

Table 1. Physico-chemical properties of glidobactins D, E, F, G and H.

	D	E	F	G	H
Nature	White powder	White powder	White powder	White powder	White powder
MP (°C, dec)	204	195	233	217	191
Molecular formula	$C_{27}H_{44}N_4O_7$	$C_{27}H_{44}N_4O_7$	$C_{25}H_{40}N_4O_6$	$C_{27}H_{44}N_4O_7$	—
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	257 (662)	260 (532)	260 (474)	257 (668)	260 (410)
IR ν_{max}^{KBr} cm^{-1}	3300, 1640, 1530	3300, 1640, 1530	3300, 1640, 1530	3300, 1640, 1530	3300, 1620, 1530
TLC[a] (Rf)	0.68	0.61	0.50	0.43	0.59
HPLC[b] (Rt; minutes)	2.2	2.6	2.8	4.4	2.4

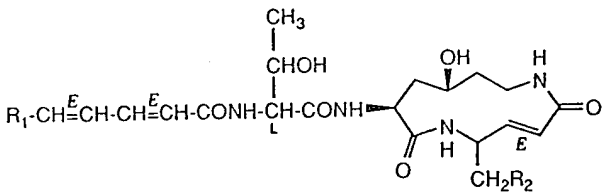
[a] Silanized, plate: EtOH - H_2O (55 : 45).
[b] SSC-ODS-262, Rt: retention time, MeOH - H_2O (4 : 1).

tive to Ryodon-Smith, iodine and sulfuric acid reactions but negative to ninhydrin, Sakaguchi, anthrone and Dragendorff tests. The molecular formulae of glidobactins D, E and G were determined to be $C_{27}H_{44}N_4O_7$, and glidobactin F as $C_{25}H_{40}N_4O_6$, by their mass and ^{13}C NMR spectra. The spectral data of glidobactin H did not allow assignment of molecular formula. The UV spectra of the five new components showed a single absorption band at 260 nm suggesting an $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl function common to glidobactins A, B and C. Their IR spectra indicated the amide carbonyl absorption at 1640 and 1530 cm^{-1}.

The physico-chemical properties obtained for glidobactins D, E, F, G and H indicated that they were closely related to the major glidobactin components. The molecular formulae assigned to glidobactins D, E and G ($C_{27}H_{44}N_4O_7$) demonstrated that they are mono-hydroxy analogs of glidobactin A ($C_{27}H_{44}N_4O_6$). Glidobactin F exhibited a C_2H_4-fewer molecular formula than that of glidobactin A. Each of the five components was hydrolyzed with 6 N HCl at 110°C for 16 hours in a sealed tube and the hydrolysate examined by TLC (BuOH - AcOH - H_2O, 3 : 1 : 1, ninhydrin detection). Glidobactins D, E, F and H afforded the same amino acids (threonine, (*E*)-4-amino-2-pentenoic acid and *erythro*-4-hydroxylysine) as glidobactin A indicating that they differed from the latter component only at the fatty acid moiety. All of these fatty acids were considered to be $\alpha,\beta,\gamma,\delta$-unsaturated acids based on their common UV maximum at 260 nm with the major antibiotics. The secondary ion mass spectrum (SI-MS) of glidobactin D yielded prominent fragment ions at *m/z* 195 (fatty acid), *m/z* 242 (glidobamine)[2] and *m/z* 296 (fatty acid-threonine) along with a protonated molecular ion at *m/z* 537. The identical molecular and fragment ions were observed in SI-MS of glidobactin E. The fatty acids from glidobactins D and E were extracted from their hydrolysates with ethyl acetate and treated with diazomethane to afford methyl esters ES-1 (from glidobactin D) and ES-2 (from glidobactin E), both of which yielded a protonated molecular ion at *m/z* 227. Since the ^1H NMR spectra had revealed that all the fatty acids of glidobactin components were straight chain acids, the acids of glidobactins D and E must be a mono-hydroxy-2,4-dodecadienoic acid. Seven sp^3 carbon signals of the fatty acid moiety of glidobactin A have been assigned as CH_3 (δ 13.4 ppm)-CH_2 (21.6)-CH_2 (30.8)-CH_2 (28.1)-CH_2 (28.0)-CH_2 (28.0)-CH_2 (31.8)[3]. A prediction of carbon shifts in substituted alkanes is well-established[4], and is used for the determination of the position of a substituent. Based on the chemical shifts of glidobactin A, calculation of the shift values of a hydroxyl group allowed us to assign the following partial structures to the fatty acids of glidobactins D and E; glidobactin D, CH_3 (δ 9.5)-CH_2 (29.5)-CHOH (70.7)-CH_2 (36.1)-CH_2 (24.5)-CH_2 (28.4)-CH_2 (31.9) and E, CH_3 (δ13.4)-CH_2 (21.7)-CH_2 (31.0)-CH_2 (24.4)-CH_2 (36.4)-CHOH (69.2)-CH_2 (40.4).

The electron impact mass spectrum (EI-MS) of glidobactin F did not give the molecular ion but showed a strong fragment ion (base peak) at *m/z* 151 which was derived from the fatty acid. Acetylation of glidobactin F in acetic anhydride and pyridine gave a diacetate derivative which gave the molecular ion at *m/z* 576 along with the fragment ions at *m/z* 151 (fatty acid) and *m/z* 285 (acetylglidobamine). Thus, glidobactin F

Fig. 1. Structures of glidobactins D, E, F and G.



Glidobactin D $R_1=CH_3CH_2CH(OH)(CH_2)_4-$ $R_2=H$

Glidobactin E $R_1=CH_3(CH_2)_4CH(OH)CH_2-$ $R_2=H$

Glidobactin F $R_1=CH_3(CH_2)_4-$ $R_2=H$

Glidobactin G $R_1=CH_3(CH_2)_6-$ $R_2=OH$

Glidobactin A $R_1=CH_3(CH_2)_6-$ $R_2=H$

Table 2. Antifungal activity of glidobactin components.

	MIC (μg/ml, SABOURAUD glucose agar)					
	D	E	F	G	H	A
Candida albicans IAM 4888	>50	>50	50	25	>50	3.1
C. albicans A9540	>50	>50	12.5	25	>50	1.6
Cryptococcus neoformans D 49	>50	>50	50	25	>50	25
Aspergillus fumigatus IAM 2530	>50	>50	25	12.5	>50	1.6
A. flavus FA 21436	>50	>50	>50	50	—	25
Fusarium moniliforme A 2284	>50	>50	>50	>50	>50	>50
Trichophyton mentagrophytes D 155	>50	>50	>50	25	>50	25

was assigned as the decadienoyl analog. The fatty acid of glidobactin H could not be determined since the antibiotic did not exhibit the molecular ion or diagnostic fragment ions in the mass spectrometry.

It has been demonstrated by the molecular formula that glidobactin G possessed an additional hydroxyl group over glidobactin A. The acid hydrolysate of glidobactin G contained threonine, *erythro*-4-hydroxylysine and an unidentified amino acid but no trace of (*E*)-4-amino-2-pentenoic acid indicating that glidobactin G differed from the other components at the (*E*)-4-amino-2-pentenoic acid moiety. This was collaborated by the ^{1}H NMR spectrum of glidobactin G which lacked the doublet methyl signal at δ 1.24 ppm attributable to (*E*)-4-amino-2-pentenoic acid. Upon enzymatic hydrolysis with papain[3], glidobactin G afforded an acidic, lipophilic substance (AC-1) and a basic, water soluble substance (BW-1). AC-1 was identical with *N*-(*E*,*E*)-2,4-dodecadienoyl-L-threonine by comparison with an authentic sample obtained from glidobactin A. The chemical ionization mass spectrum (CI-MS) of BW-1 showed the protonated molecular ion at *m/z* 258, 16 mass units higher than the corresponding moiety of glidobactins A, B and C. Thus, glidobactin G contains (*E*)-4-amino-5-hydroxy-2-pentenoic acid in place of the (*E*)-4-amino-2-pentenoic acid of glidobactin A. The structures of glidobactins D, E, F and G are shown in Fig. 1.

All five new components of glidobactin did not exhibit significant activity against Gram-positive and Gram-negative bacteria. Glidobactins F and G were slightly active against fungi but activity was much weaker than that of glidobactin A (Table 2).

Antitumor activity of glidobactins D, E, F, G and H was examined for mouse P388 leukemia following the method described in the previous report.[1] Glidobactins F and G showed significant prolongation of the life span at 3 mg/kg/day but the other three components were inactive at the doses tested (0.3~3 mg/kg/day). The antitumor activity of glidobactins F and G

was weaker than that of glidobactin A which was active at 0.3 and 1 mg/kg/day.

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GLIDOBACTINS A, B AND C, NEW ANTITUMOR ANTIBIOTICS

II. STRUCTURE ELUCIDATION

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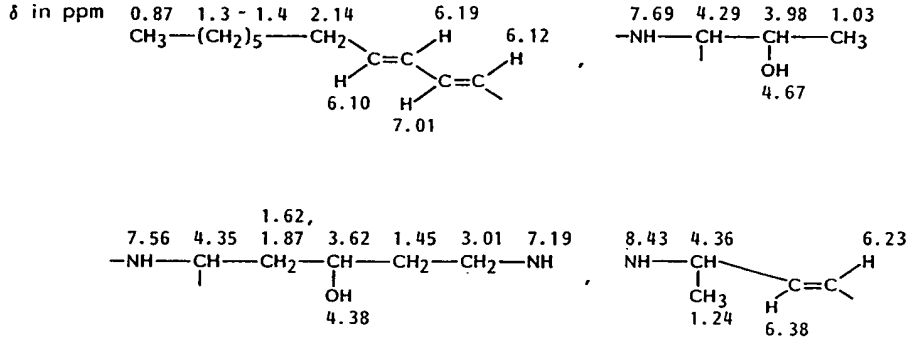
The structures of new antitumor antibiotics, glidobactins A (**Ia**), B (**Ib**) and C (**Ic**) were elucidated by a combination of chemical and enzymatic degradations and spectral analyses. They have in common a cyclized tripeptide nucleus composed of L-threonine, 4(*S*)-amino-2(*E*)-pentenoic acid and *erythro*-4-hydroxy-L-lysine, and differ from each other in the unsaturated fatty acid moiety attached to the peptide.

In the course of continuing search for novel antitumor antibiotics in the microbial metabolites, *Polyangium brachysporum* sp. nov. No. K481-B101 (ATCC 53080) collected in Greece was found to produce novel antibiotic complex with antifungal and antitumor activity[1~3]. The antibiotic complex named glidobactin was extracted and separated into three active components, glidobactins A (**Ia**), B (**Ib**) and C (**Ic**). In addition to their broad antifungal activity, all the components exhibited potent antitumor activity against P388 leukemia implanted in mice with the T/C values in the range of 200 to 250%. In this report, we present structural studies of **Ia**, **Ib** and **Ic**, which have shown unique acylated 12 membered cyclic peptide structures.

Spectral Characteristics

Glidobactins A (**Ia**), B (**Ib**) and C (**Ic**) were isolated from the fermentation broth of strain K481-B101 by butanol extraction followed by column chromatographies on silica gel and reversed phase silica gel. **Ia** and **Ic** were obtained as colorless needles from aqueous methanol and methanol, respectively, while **Ib** was isolated as crystalline powder. **Ia**: $C_{27}H_{44}N_4O_6$; *m/z* 520 (M^+); mp 259~261°C; $[\alpha]_D^{24}$ −111° (*c* 0.5, MeOH). **Ib**: $C_{29}H_{46}N_4O_6$; *m/z* 546 (M^+); mp 232~234°C; $[\alpha]_D^{24}$ −92° (*c* 0.5, MeOH). **Ic**: $C_{29}H_{48}N_4O_6$; *m/z* 548 (M^+); mp 273~275°C; $[\alpha]_D^{24}$ −104° (*c* 0.5, MeOH). The UV spectra of the three components exhibited the same absorption maximum at 261 nm in methanol suggesting the presence of an $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl functionality. The IR absorption at around 1630 and 1540 cm^{-1} indicated amide group in their molecules, as described in the preceding paper[3].

The ^{13}C NMR spectrum of **Ia** displayed 26 carbon signals with one of them being double intensity (δ 28.0). The off-resonance experiments allowed to assign these signals as three methyl (δ 13.4, 18.2 and 19.4), nine methylene (δ 21.6, 28.0, 28.1, 30.8, 31.8, 39.3, 39.4 and 41.7), five methine (δ 44.7, 51.1, 57.9, 66.2 and 66.4), six doublet sp^2 (δ 122.8, 123.0, 128.6, 139.4, 141.5 and 142.6) and four singlet sp^2 assignable to amide (δ 165.5, 167.4, 169.0 and 170.8) carbons. This was ascertained from the ^1H NMR spectrum which showed the presence of three methyl (δ 0.87 (t), 1.03 (d) and 1.24 (d)), six vinyl (d, all of them being splitted by *trans* coupling), four amide (δ 7.19, 7.56, 7.69



and 8.43) and two hydroxyl protons (δ 4.38 and 4.67) in addition to 23 other methylene or methine protons in around δ 1.3~4.7.

The detail ^{1}H NMR analyses (Table 1) assisted by the ^{1}H-^{1}H 2D correlated spectroscopy (COSY) carried out for **Ia** provided the above 4 structural fragments in the molecule.

The ^{1}H and ^{13}C NMR spectra of **Ib** and **Ic** were very similar to those of **Ia** differing by the presence of an additional –CH=CH– and $-CH_2CH_2-$ unit, respectively, to **Ia**. The spectral differences reflected the differences in the molecular formulae among **Ia**, **Ib** and **Ic**.

The presence of two hydroxyl groups in **Ia** was revealed by the fact that acetylation in pyridine afforded di-*O*-acetyl derivative (**IIa**: *m/z* 604 (M^+)). When hydrogenated over palladium on charcoal, **Ia** yielded hexahydroglidobactin A (**III**), whose ^{1}H NMR indicated the absence of the six *trans*-double bond protons observed in the spectrum of **Ia** and increased protons at around δ 1.3~3.0.

Constituent Amino Acids and Fatty Acid

Ia was heated with 6 N HCl under reflux for 16 hours. After removal of the lipophilic product (**VIII**) by ethyl acetate extraction, the hydrolysate was concentrated to an oily residue which was chromatographed on Dowex 50W-X4 to obtain L-threonine (**IV**: $[\alpha]_D^{25}$ −12.7°) and three unusual amino acids (**V**, **VI** and **VII**). Amino acid **V** was identified as a mixture of diastereoisomers of 4-amino-3-hydroxy-*n*-valeric acid by its ^{1}H NMR, electron impact (EI)-MS (*m/z* 134 $(M+H)^+$) and a direct comparison with the authentic sample[4]. The molecular formula of **VI** was assigned to be $C_5H_9NO_2$ by elemental analysis and EI-MS (*m/z* 115 (M^+)). Its ^{1}H NMR showed the presence of one methyl δ 1.50 (d, J=6.2 Hz), one methine δ 4.0~4.2 (m) and two *trans* olefinic protons δ 6.05 (d, J=15.3 Hz) and 6.57 (dd, J=6.2 and 15.3 Hz). These spectral data combined with the specific rotation ($[\alpha]_D^{22.5}$ −6° in 5 N HCl) indicated **VI** to be 4-(*S*)-amino-2(*E*)-pentenoic acid[5]. Dehydration of compound **V** with concentrated sulfuric acid afforded compound **VI**[5]. Amino acid **VII** was determined to be 4-hydroxylysine based on its elemental analysis ($C_6H_{14}N_2O_3$), EI-MS (*m/z* 163 $(M+H)^+$) and ^{1}H NMR spectrum (δ 1.8~2.1, 4H, m, 3.18, 2H, t and 3.6~4.3, 2H, m) and also by formation of a γ-lactone compound ($\nu_{C=O}$ 1770 cm^{-1}) upon treatment with 6 N HCl. The amino acid was deoxygenated by hydroiodic acid and red phosphorus, and the product was treated with 2,4-dinitrofluorobenzene. The di-DNP derivative obtained was identical with authentic di-DNP-L-lysine by TLC and IR and CD spectra[6]. IZUMIYA *et al.* reported mutarotation of 4-hydroxy-lysines[7] and the shift observed for **VII** (Δ −37° from the lactone form to the free amino acid form) clearly indicated *erythro*-L-configuration.

The lipophilic acidic fraction (VIII) obtained in the above acid hydrolysis showed the UV maximum at 258 nm (ε 24,000) and a carboxyl absorption (1680 cm^{-1}) in the IR spectrum. Upon treatment with diazomethane, VIII yielded a monomethyl derivative VIIIe (*m/z* 210 (M^+), λ_{max}^{MeOH} nm 260). These data coupled with their ^1H and ^{13}C NMR spectra indicated 2,4-dodecadienoic acid for VIII. The observed coupling constants (J=16.0 Hz) made it obvious that both double bonds are *trans* configuration[6].

Ia has been given a molecular formula of $C_{27}H_{44}N_4O_6$ and exhibited six olefinic carbons and four amide carbons in the ^{13}C NMR. In addition, the ^1H NMR of Ia did not exhibit the structural fragment assignable to V. Thus, amino acid V was considered as an artifact produced by hydration of the natural amino acid VI during the acid hydrolysis.

IV: L-Threonine; $CH_3CHCHCOOH$ (OH, NH_2)

V: 4-Amino-3-hydroxy-*n*-valeric acid; $CH_3CH\ CHCH_2COOH$ (NH_2, OH)

VI: 4(*S*)-Amino-2(*E*)-pentenoic acid; $CH_3CHCH{=}CHCOOH$ (NH_2)

VII: *Erythro*-4-hydroxy-L-lysine; $NH_2CH_2CH_2CHCH_2CHCOOH$ (OH, NH_2)

VIII: 2(*E*),4(*E*)-Dodecadienoic acid; $CH_3(CH_2)_6CH{=}CHCH{=}CHCOOH$

Mild Acid Hydrolysis

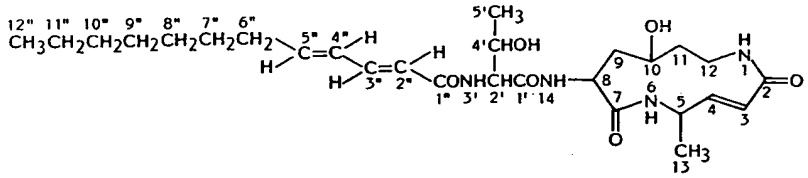
Upon hydrolysis with 6 N HCl at 35°C for 3 hours, Ia was degraded to a bioinactive compound (IX, isolated as sodium salt: $C_{27}H_{43}N_4O_7Na$, secondary ion (SI)-MS, (*m/z* 561 $(M+H)^+$) which produced the same amino acids IV, V, VI and VII and fatty acid (VIII) as those of Ia upon complete acid hydrolysis. When treated with acetic anhydride and pyridine, IX afforded an acetyl-γ-lactone compound ($\nu_{C=O}$ 1770 cm^{-1}). Reaction of IX with 2,4-dinitrofluorobenzene followed by acid hydrolysis of the resulting DNP-derivative yielded 4-DNP-amino-2-pentenoic acid (DNP-VI) together with free amino acids IV and VII. These results evidenced that the mild hydrolysis cleaved only one of the peptide bonds of Ia producing a straight chain peptide with amino acid VI at the *N*-terminal.

Enzymatic Hydrolysis

Glidobactin A was cleaved by papain to yield an acidic, lipophilic compound (X) and a basic water-soluble compound (XI). Compound X was shown to be 2,4-dodecadienoylthreonine (VIII~IV) by its IR ($\nu_{C=O}$ 1720 and 1650 cm^{-1}), chemical ionization (CI)-MS (*m/z* 298 $(M+H)^+$ and *m/z* 179 (M^+-IV)) and ^1H NMR spectrum (Table 1). The structure was further substantiated by the fact that X yielded IV and VIII on hydrolysis in 6 N HCl.

When refluxed in 6 N HCl, compound XI afforded amino acid V, VI and VII as revealed by TLC. In the EI-MS of XI, the molecular ion was observed at *m/z* 241 indicating a cyclic peptide composed of VI and VII. In accordance with this assumption, the ^{13}C NMR measured at pD 8.0 (Table 2) exhibited two carbonyl, two vinyl, three methine, three methylene and one methyl carbon signals. When the spectrum was measured at pD 2.0, the protonation shifts were observed on a carbonyl (δ 175.3, Δ 6.1 ppm) and a methylene carbons (δ 44.6, Δ 4.8 ppm) indicating the twelve membered cyclic peptide

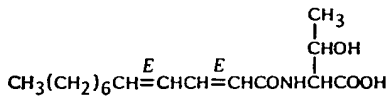
Table 1. The ^{1}H NMR of glidobactin A (**Ia**) and enzymatic hydrolysis products (**X** and **XI**) in DMSO-d_6.



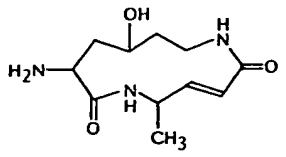
Position No.	Ia	X	XI
1	7.19 (dd, J=5.7, 7 Hz)		7.37 (dd, J=5.3, 6.9 Hz)
3	6.23 (d, J=15.9 Hz)		6.17 (dd, J=1, 15.9 Hz)
4	6.38 (dd, J=5.9, 15.9 Hz)		6.45 (m)
5	4.36 (m)		4.3 (m)
6	8.43 (d, J=7.5 Hz)		8.35 (d, J=7.2 Hz)
8	4.35 (m)		3.26 (m)
9	1.87 (ddd, J=6.6, 7, 13.5 Hz),		1.74 (m),
	1.62 (ddd, J=3.7, 3.8, 13.5 Hz)		1.53 (m)
10	3.62 (m)		3.48 (m)
10-OH	4.38 (br s)		4.5 (br s)
11	1.45 (m)		1.35 (m)
12	3.01 (m)		3.0 (m)
13	1.24 (d, J=7 Hz)		1.24 (m)
14	7.56 (d, J=7.6 Hz)		—
2′	4.29 (dd, J=4.1, 8.7 Hz)	4.15 (dd, J=3, 7.4 Hz)	
3′	7.69 (d, J=8.6 Hz)	7.55 (d, J=8.1 Hz)	
4′	3.98 (m)	3.92 (m)	
4′-OH	4.67 (br s)	—	
5′	1.03 (d, J=6.2 Hz)	0.92 (d, J=6.6 Hz)	
2″	6.12 (d, J=15.2 Hz)	6.16 (d, J=15 Hz)	
3″	7.01 (dd, J=10.4, 15.2 Hz)	6.96 (dd, J=10, 15 Hz)	
4″	6.19 (dd, J=10, 15.2 Hz)	6.17 (dd, J=10, 15 Hz)	
5″	6.1 (dt, J=15.2, 6.6 Hz)	6.04 (dt, J=15, 6.6 Hz)	
6″	2.14 (dt, J=6.6, 6.8 Hz)	2.11 (dt, J=5, 6.6 Hz)	
7″	1.4 (m)	1.37 (m)	
8″-11″	1.3 (m)	1.25 (m)	
12″	0.87 (t, J=7 Hz)	0.86 (t, J=7.3 Hz)	

Ia (400 MHz, at 70°C), **XI** (400 MHz, at 23°C) and **X** (270 MHz, at 23°C).

structure shown below. The ^{1}H NMR (Table 1) exhibited two amide NH protons at δ 7.37 (dd) and 8.35 (d) supporting the assigned structure. Further support was obtained by the mono-*N*-acetyl-**XI** (*m/z* 283 (M^+)) which exhibited two doublet amide (δ 7.98 and 8.57) and one triplet amide protons (δ 7.34).



Compound **X**

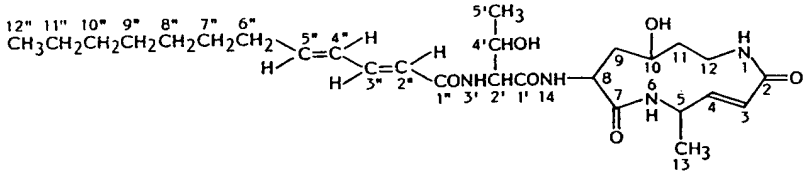


Compound **XI**

Structure of Glidobactin A (Ia)

As discussed in the preceding paper[3], glidobactin A (**Ia**) is negative to ninhydrin reaction and did not exhibit ester carbonyl absorption

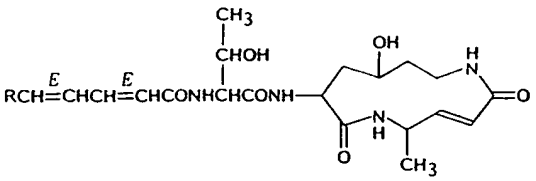
Table 2. The ^{13}C NMR of glidobactin A (Ia) and enzymatic hydrolysis products (X and XI).



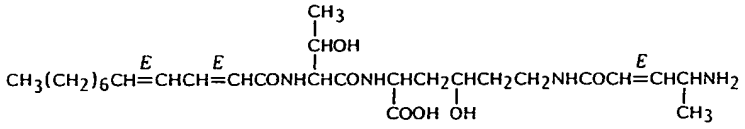
Position No.	Ia	X	XI	Position No.	Ia	X	XI
2	167.4 (s)		172.0 (s)	5′	19.4 (q)	20.0 (q)	
3	123.0 (d)		122.9 (d)	1″	165.5 (s)	165.6 (s)	
4	142.6 (d)		146.6 (d)	2″	122.8 (d)	123.5 (d)	
5	44.7 (d)		46.7 (d)	3″	139.4 (d)	139.5 (d)	
7	170.8 (s)		176.7 (s)	4″	128.6 (d)	128.7 (d)	
8	51.1 (d)		54.0 (d)	5″	141.5 (d)	141.5 (d)	
9	41.7 (t)		44.6 (t)	6″	31.8 (t)	32.3 (t)	
10	66.2 (d)		68.9 (d)	7″	28.0[a](t)	28.6[c](t)	
11	39.3[b](t)		39.0 (t)	8″	28.0[a](t)	28.6[c](t)	
12	39.4[b](t)		41.0 (t)	9″	28.1[a](t)	28.5[c](t)	
13	18.2 (q)		18.5 (q)	10″	30.8 (t)	31.3 (t)	
1′	169.0 (s)	173.3 (s)		11″	21.6 (t)	22.1 (t)	
2′	57.9 (d)	58.2 (d)		12″	13.4 (q)	13.8 (q)	
4′	66.4 (d)	66.8 (d)					

[a~c] Assignment may be interchanged.
Ia (100 MHz, DMSO-d_6, at 70°C), X (20 MHz, DMSO-d_6, at 23°C), XI (20 MHz, D_2O at pH 8.0, at 23°C).

Fig. 1. Structures of glidobactins A (Ia), B (Ib) and C (Ic) and IX.



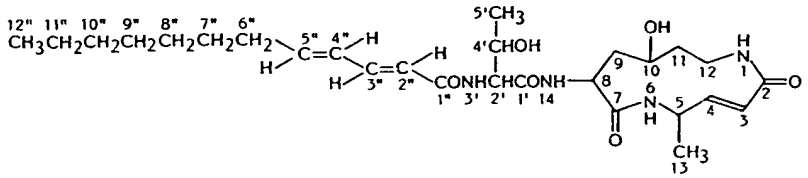
Glidobactin A (**Ia**) R = $CH_3(CH_2)_6$-
Glidobactin B (**Ib**) R = $CH_3(CH_2)_4CH$=$CH(CH_2)_2$- (Z)
Glidobactin C (**Ic**) R = $CH_3(CH_2)_8$-



Peptide **IX**

in the IR spectrum. Thus, joining X and XI by a peptide bond naturally provides the structure of glidobactin A (Fig. 1). Compound X was coupled with 1-hydroxy-1,2,3-benzotriazole (HOBT) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) to give HOBT ester which was reacted with XI in dimethylformamide. The product, after purification, was identical in all respects with glidobactin A (Ia).

Table 3. Long range ^{1}H-^{13}C correlation of glidobactin A (Ia)[a].



Chemical shift of C=O carbon (ppm)	C-H proton correlated (ppm)	N-H proton correlated (ppm)	Assignment of C=O carbon
165.5	7.01, 6.19	7.69	1″
167.4	6.23	—	2
169.0	4.29	7.56	1′
170.8	1.62	8.43	7

[a] In DMSO-d_6, 400 MHz for proton and 100 MHz for ^{13}C at 70°C.
—: No connectivity was observed.

The data obtained for **IX** are consistent with the linear peptide structure (Fig. 1) which was produced by cleavage of the peptide between **VI** and **VII**. As described latter part, **IX** was cyclized to yield the original antibiotic **Ia** by treatment with HOBT and DCC.

The ^{1}H and ^{13}C NMR spectra of **Ia** were analyzed as shown in Tables 1 and 2. The assignments were made on the basis of ^{1}H-^{1}H 2D and ^{1}H-^{13}C 2D COSY experiments. The ^{1}H-^{13}C COSY *via* small coupling optimized for J=8.0 Hz made the assignments of four amide carbonyl carbons possible as they gave correlation peaks to a neiboring C-H as well as an amide NH proton in a different spin system (Table 3). The assignments are consistent with the proposed structure of **Ia**.

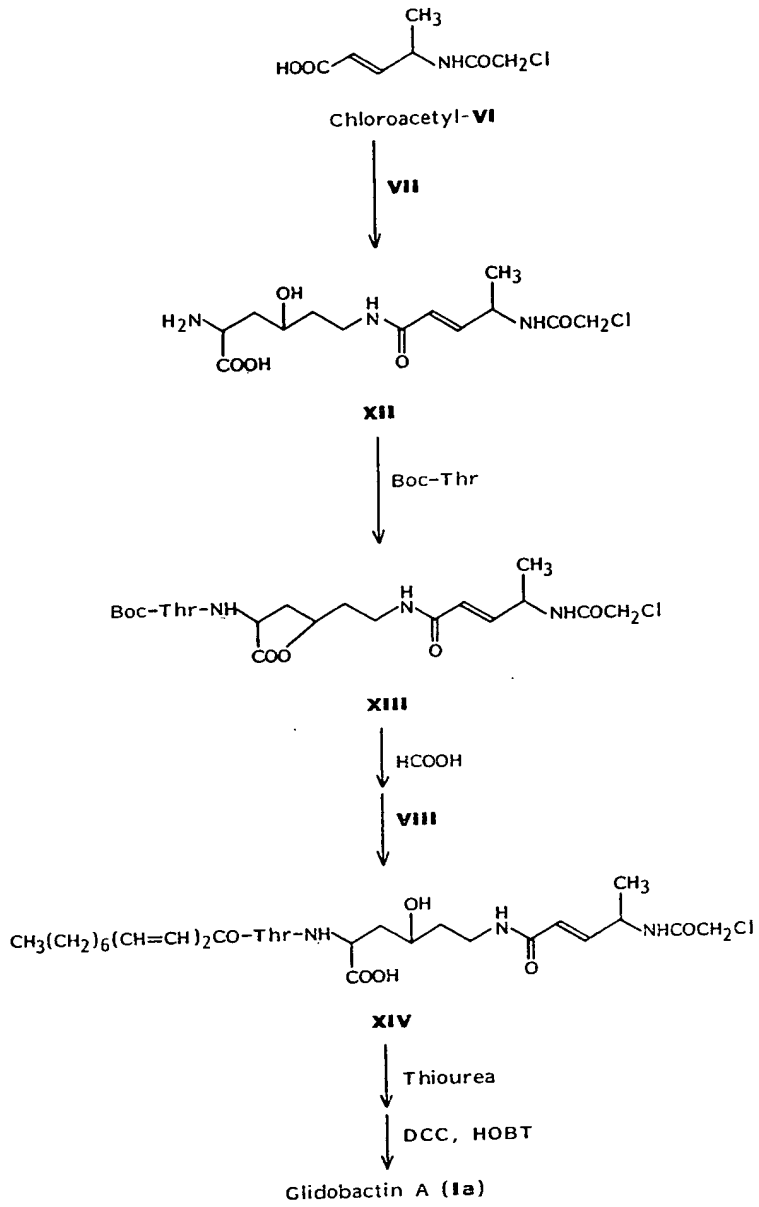
Synthesis of Glidobactin A (Ia)

Treatment of 4-amino-2(*E*)-pentenoic acid (**VI**) with chloroacetyl chloride in dimethylformamide gave *N*-chloroacetyl-**VI** which was coupled with *erythro*-4-hydroxy-L-lysine (**VII**) using the HOBT-methodology. The structure of the product (**XII**) was established to be $^{\varepsilon}N$-acyl derivative based on the ^{1}H NMR which exhibited a doublet (8.42 ppm) and a double-doublet amide (8.05 ppm) protons. Acylation of **XII** with the active ester of BOC-L-threonine afforded fully protected tripeptide lactone (**XIII**). Removal of the BOC group of **XIII** followed by acylation with 2(*E*),4(*E*)-dodecadienoic acid (**VIII**) using HOBT-active ester method gave **XIV** in 50% yield. The chloroacetyl group of **XIV** was removed by thiourea to yield the straight chain peptide which was identical with **IX** in their physicochemical and spectral properties. Compound **IX** was treated with HOBT and DCC in dimethylformamide and the products were purified by reversed phase silica gel chromatography. One of the products isolated was bioactive and identified as glidobactin A by a direct comparison with the natural antibiotic (Scheme 1).

Structures of Glidobactins B (Ib) and C (Ic)

On heating with 6 N HCl, **Ib** and **Ic** afforded the same amino acid complex as that obtained from **Ia**. The fatty acids of the antibiotics (**XV** from **Ib** and **XVI** from **Ic**) were extracted from the hydrolysates and converted to methyl esters (**XVe**: *m/z* 236 (M^+) and **XVIe**: *m/z* 238 (M^+)). The UV (λ_{max} 260 nm) and EI-MS of **XVe** and **XVIe** were similar to those of **VIIIe** (methyl 2,4-dodecadienoate), and the differences observed in their spectral data indicated that **XVe** and **XVIe** had an additional

Scheme 1. Synthesis of glidobactin A.



CH=CH and CH_2CH_2 moiety to **VIIIe**, respectively, reflecting the differences of molecular formulae between glidobactins B (**Ib**) and C (**Ic**) and glidobactin A (**Ia**). Ozonolysis of **Ib** followed by reductive degradation of the ozonide yielded *n*-hexanal which was identified as 2,4-dinitrophenylhydrazone (*m/z* 280 (M^+)). The extensive decoupling studies enabled the assignment of *Z* geometry for the additional double bond of **Ib** (J=11.0 Hz). Thus, **XVe** and **XVIe** are methyl 2(*E*),4(*E*),8(*Z*)-tetradecatrienoate and methyl 2(*E*),4(*E*)-tetradecadienoate, respectively and **XV** and **XVI** are the corresponding free acids. Hence, the structures of **Ib** and **Ic** are represented as shown in Fig. 1.

Discussion

The structures of glidobactins A, B and C were established to be quite unique acylpeptides having an unusual twelve membered cyclic ring (Fig. 1). The twelve membered ring peptide is composed of two amino acids, 4(*S*)-amino-2(*E*)-pentenoic acid and *erythro*-4-hydroxy-L-lysine, both of which have never been found in the natural source. The three components differ only in the length and/or unsaturation of the fatty acid attached to their common peptide core. As described in the preceding paper[3], glidobactins A, B and C exhibit strong antifungal and antitumor activities with the potency being slightly different among the three components. Both hexahydroglidobactin A and the linear peptide compound **IX** are completely bio-inactive indicating the importance of the double bonds and the cyclic structure of the antibiotics. On the other hand, diacetylglidobactin A retains antifungal and antitumor activity. The structure-activity relationship observed above suggested that the chemical or biological modifications of this rather simple molecule may yield derivatives with improved activity. Manuscripts detailing the modification studies are in preparation and will be submitted to this journal.

Experimental

TLC was carried out on precoated silica gel plates (Kieselgel $60F_{254}$, Merck, layer thickness 0.25 mm). The IR spectra were determined on a Jasco IRA-1 spectrometer and the UV spectra on a Shimadzu UV-200. The ^1H NMR and ^{13}C NMR spectra were recorded on a Jeol C-60HL, a Varian Model FT 80A, a Jeol FX 270, a Bruker WM-360 or a Jeol GX400 using tetramethylsilane as a standard. The ordinary mass spectra were obtained with a Hitachi RMU-6MG mass spectrometer modified with an in-beam/electron impact system, the high-resolution (HR) spectra on a Hitachi-M80 mass spectrometer, the SI-MS on a Hitachi M-80B (Xenon, 8 KeV) and CI-MS on a Jeol JMS-DX 300 with NH_3 as reagent gas. Optical rotations were determined with a Jasco model DIP 140.

Acetylation of Glidobactins A (Ia), B (Ib) and C (Ic)

A mixture of glidobactin A (**Ia**, 100 mg), acetic anhydride (4 ml) and pyridine (4 ml) was stirred overnight at room temperature. The mixture was poured into ice water (15 ml) and the solution was extracted with EtOAc (3×15 ml). The combined extracts were washed with water (10 ml), dried over sodium sulfate and evaporated to give crude solid (97 mg). This was chromatographed on a silica gel column (Wakogel C-200, 60 ml), developing with $CHCl_3$ and then with MeOH - $CHCl_3$ (5 : 95). The eluate was monitored by TLC ($CHCl_3$ - MeOH, 9 : 1), and the fractions containing the major product (Rf 0.29) were combined and evaporated to give 48 mg of **IIa** as colorless powder. **IIa**: MP 197~198°C; $[\alpha]_D^{24}$ −117° (*c* 0.5, MeOH); EI-MS *m/z* 604 (M^+); UV λ_{max}^{MeOH} nm (ε) 261 (42,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1745, 1650, 1540.

Anal Calcd for $C_{31}H_{49}N_4O_8 \cdot H_2O$: C 59.78, H 8.09, N 9.00.
Found: C 59.78, H 7.93, N 8.89.

Similarly, acetylation of **Ib** (20 mg) and **Ic** (35 mg) yielded **IIb** (11 mg) and **IIc** (28 mg), respectively. **IIb**: MP 176~178°C; $[\alpha]_D^{23}$ −99° (*c* 0.5, MeOH); EI-MS *m/z* 630 (M^+); UV λ_{max}^{MeOH} nm (ε) 261 (48,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1740, 1650, 1540.

Anal Calcd for $C_{33}H_{50}N_4O_9 \cdot H_2O$: C 61.09, H 8.08, N 8.64.
Found: C 60.64, H 8.18, N 8.43.

IIc: MP 199~201°C; $[\alpha]_D^{24}$ −107° (*c* 0.5, MeOH); EI-MS *m/z* 632 (M^+); UV λ_{max}^{MeOH} nm (ε) 261 (47,000), IR ν_{max}^{KBr} cm^{-1} 3300, 1740, 1645, 1540.

Anal Calcd for $C_{33}H_{52}N_4O_9 \cdot \frac{1}{2}H_2O$: C 61.76, H 8.32, N 8.73.
Found: C 61.45, H 8.28, N 8.48.

Hydrogenation of Ia

Ia (200 mg) in BuOH (20 ml), EtOH (10 ml) and water (10 ml) mixture was hydrogenated at 3 kg/cm^2 over 10% palladium on charcoal in a Parr equipment for 6 hours. After the catalyst was removed by filtration, the filtrate was evaporated *in vacuo* to a residue which was triturated with hot 50% aqueous EtOH (10 ml). The insolubles were collected by filtration and dried *in vacuo* to give

177 mg of white powder of **III**: MP 271°C; HREI-MS *m/z* 526.3757 (calcd for $C_{27}H_{50}N_4O_6$: 526.3727); UV end absorption.

Acid Hydrolysis of Ia

A suspension of **Ia** (200 mg) in 6 N HCl (9 ml) was refluxed for 16 hours and the resulting solution was concentrated *in vacuo* to dryness. The residue was vigorously shaken with a mixture of water (40 ml) and EtOAc (30 ml). Evaporation of the EtOAc layer yielded brown oil (74 mg) containing **VIII**. The aqueous layer was concentrated *in vacuo* to an oil (210 mg) which was chromatographed on a column of Dowex 50W-X4 (70 ml). The column was developed with 0.1 M pyridine - formic acid (pH 3.1), 0.2 M pyridine - formic acid (pH 3.1) and 0.2 M pyridine - acetic acid (pH 4.4), successively, and the eluate was monitored by ninhydrin test and TLC (MeOH - 10% $AcONH_4$ - 10% NH_4OH, 10:9:1). **IV** was eluted by 0.1 M pyridine - formic acid (pH 3.1), **V** and **VI** were by 0.2 M pyridine - formic acid (pH 3.1) in that order and **VII** was by 0.2 M pyridine - AcOH (pH 4.4). Evaporation of the appropriate fractions gave nearly pure solids of **IV**, **V**, **VI** and **VII**. **VI** was desalted with Sephadex LH-20 chromatography eluting with 50% aqueous MeOH and **IV**, **V** and **VII** were again purified by Dowex 50W-X4 and then desalted. **IV**: Colorless rods from aqueous EtOH (38 mg); mp 244°C; $[\alpha]_D^{25.5}$ −12.7° (*c* 0.7, 5 N HCl); ^1H NMR (60 MHz, D_2O) δ 1.46 (3H, d, J=7.0 Hz), 4.07 (1H, J=5.4 Hz), 4.4 (1H, m). **V**: White amorphous powder (20 mg); $[\alpha]_D^{26}$ −3.3° (*c* 0.3, 2 N HCl); EI-MS *m/z* 134 $(M+H)^+$; IR ν_{max}^{KBr} cm^{-1} 3400, 1720, 1610; ^1H NMR (60 MHz, D_2O) δ 1.34 (*ca.* 1.5H, d, J=7.5 Hz), 1.38 (*ca.* 1.5H, d, J=7.5 Hz), 2.4~2.6 (2H, m), 3.1~3.7 (1H, m), 3.8~4.4 (1H, m). **VI**: Colorless needles from aqueous EtOH (8 mg); mp 223~224°C (literature[5] 221.5~223.5°C); $[\alpha]_D^{24.5}$ −6° (*c* 1.0, 5 N HCl) (literature $[\alpha]_D^{22.5}$ −5.8°); EI-MS *m/z* 115 (M^+); IR ν_{max}^{KBr} cm^{-1} 1630, 1580; ^1H NMR (60 MHz, D_2O) δ 1.50 (3H, d, J=6.2 Hz), 4.0~4.2 (1H, m), 6.05 (1H, d, J=15.3 Hz), 6.57 (1H, dd, J=6.2 and 15.3 Hz).

Anal Calcd for $C_6H_9NO_2$: C 52.16, H 7.88, N 12.17.
Found: C 51.99, H 8.12, N 12.06.

VII: Colorless needles from aqueous EtOH containing conc HCl (16 mg); mp 223~225°C (literature[7] 206~207°C); $[\alpha]_D^{26}$ +23° (*c* 0.79, 6 N HCl) (literature $[\alpha]_D^{20}$ +23.6°); EI-MS *m/z* 163 $(M+H)^+$; IR ν_{max}^{KBr} cm^{-1} 3300, 1640, 1590, 1560; ^1H NMR (60 MHz, D_2O) δ 1.8~2.1 (4H, m), 3.18 (2H, t, J=8.2 Hz), 3.6~4.3 (2H, m).

Anal Calcd for $C_6H_{14}N_2O_3 \cdot HCl$: C 36.28, H 7.61, N 14.10, Cl 17.85.
Found: C 36.13, H 7.62, N 13.74, Cl 18.07.

The oily residue (74 mg) obtained from the EtOAc extract was chromatographed on a silica gel column (100 ml) developed with $CHCl_3$ - MeOH (99:1). The UV-absorbing fractions were collected and evaporated to afford 29 mg of oil, which was further purified by preparative TLC developed by $CHCl_3$ - MeOH (9:1). The appropriate band (Rf 0.49) was collected and eluted with the developing solvent mixture. Evaporation of the eluate gave 16 mg of oil which was chromatographed on Sephadex LH-20 and crystallized to give colorless needles of **VIII** (10 mg): MP 48~49°C (literature[8] 49~51°C); UV λ_{max}^{MeOH} nm (ε) 258 (24,000); IR ν_{max}^{KBr} cm^{-1} 1680, 1630, 1605; ^1H NMR (80 MHz, DMSO-d_6) δ 0.89 (3H, t, J=6.2 Hz), 1.3 (10H, m), 2.23 (2H, m), 5.73 (1H, d, J=15.0 Hz), 6.18 (2H, m), 7.14 (1H, m); ^{13}C NMR (20 MHz, DMSO-d_6) δ 14.0 (q), 22.3 (t), 28.5 (t), 28.7 (t), 28.8 (t), 31.5 (t), 32.5 (t), 120.4 (d), 128.5 (d), 143.8 (d), 144.5 (d), 167.8 (s). **VIII** (7 mg) in ether (5 ml) was treated with excess amount of diazomethane at room temperature. After 3 hours, the reaction mixture was washed with aqueous $NaHCO_3$ (1 ml), water (1 ml), 10% HCl (1 ml) and water (1 ml), successively. Evaporation of the dried ether extract gave 5 mg of oil (**VIIIe**); EI-MS *m/z* 210 (M^+), 179 $(M^+ - OCH_3)$; UV λ_{max}^{MeOH} nm (ε) 260 (22,000).

Acid Hydrolysis of Ib and Ic

Ib and **Ic** (20 mg each) were hydrolyzed with 6 N HCl (0.5 ml) under a similar condition as used in the hydrolysis of **Ia**. The reaction mixture was separated into a lipophilic acidic substance and water-soluble amino acid complex. The acidic product was treated with excess of diazomethane and the residual ester was purified by preparative TLC ($CHCl_3$). **XVe** (1.0 mg from **Ib**): EI-MS *m/z* 236 (M^+); UV λ_{max}^{MeOH} nm (ε) 260 (20.000). **XVIe** (1.5 mg from **Ic**): EI-MS *m/z* 238 (M^+); UV λ_{max}^{MeOH} nm

(ε) 260 (19,000). The amino acid complex from **Ib** and **Ic** was identical with that from **Ia** by TLC (IV, V, VI and VII).

Mild Hydrolysis of **Ia**

A suspension of **Ia** (432 mg) in 6 N HCl (43 ml) was stirred at 35°C for 3 hours. The solution was adjusted to pH 7 and extracted with BuOH (2×40 ml). The combined extracts were evaporated to dryness (pale yellow solid, 500 mg) which was chromatographed on a column of Sephadex LH-20 (800 ml) eluting with 50% aqueous MeOH. The eluate was monitored by TLC (BuOH - AcOH - H_2O, 3 : 1 : 1; Rf 0.38) and the fractions containing the major product were combined and evaporated to give white amorphous powder (85 mg). This solid (50 mg) was dissolved in water (3 ml, pH 9) and chromatographed on a reversed phase silica gel (Merck Silica gel 60 silanised, 50 ml) column eluting with aqueous MeOH (50 and 70%). The ninhydrin-positive fractions were combined, evaporated *in vacuo* and lyophilized to afford 45 mg of **IX**·Na salt: MP >155°C (dec); $[\alpha]_D^{24}$ −16° (*c* 0.5, H_2O); SI-MS *m/z* 561 $(M+H)^+$; UV λ_{max}^{MeOH} nm (ε) 260 (32,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1630, 1610, 1530.

Anal Calcd for $C_{27}H_{43}N_4O_7Na \cdot H_2O$: C 56.04, H 8.19, N 9.68.
Found: C 55.75, H 8.32, N 9.44.

Determination of the terminal amino acid was carried out by the following proceure: A mixture of **IX** (2.5 mg), 2,4-dinitrofluorobenzene (2.5 mg) and $NaHCO_3$ (5 mg) in 50% aqueous EtOH (0.5 mg) was stirred for 1 hour and then concentrated to dryness. The residue was partitioned between water (1 ml) and ether (1 ml). The aqueous layer was acidified to pH 2 and extracted with $CHCl_3$ (2×2 ml). After evaporation of the extracts, the residue was purified by preparative TLC ($CHCl_3$ - MeOH, 9 : 1). The major yellow band (Rf 0.5) was extracted with $CHCl_3$ and the extract was concentrated to give 2 mg of DNP-IX. The DNP-IX was hydrolyzed with 6 N HCl (1 ml) at 100°C overnight. EtOAc extraction of the hydrolysate yielded yellow solid of DNP-**VI** (1 mg) which was identified with an authentic sample prepared by dinitrophenylation of **VI**. DNP-**VI**: ^1H NMR (360 MHz, $CDCl_3$) δ 1.63 (3H, d, *J*=5.5 Hz), 4.48 (1H, m), 5.98 (1H, dd, *J*=1.5 and 16.2 Hz), 6.80 (1H, d, *J*=6.5 Hz), 7.04 (1H, dd, *J*=16.2 and 5.5 Hz), 8.28 (1H, dd, *J*=2.1 and 6.5 Hz), 8.57 (1H, br d, *J*=6.5 Hz), 9.19 (1H, d, *J*=2.1 Hz).

Hydrolysis of **Ia** with Papain

A mixture of **Ia** (4 g) and papain (Sigma P-3375, 50 g) in 20 liters of 10% aqueous MeOH was stirred at 28°C for 22 hours. The mixture was acidified to pH 3.3 by AcOH and extracted with EtOAc (10 liters). Evaporation of the extract afforded an oil (6.3 g) which was chromatographed on silica gel (250 ml) with a mixture of CH_2Cl_2 and MeOH (9 : 1) to give a semi-pure oily compound **X** (3.3 g). Further chromatographic purification of the oil on Sephadex LH-20 followed by crystallization gave colorless needles of **X**, 1.15 g (yield 50%): MP 90~91°C; $[\alpha]_D^{27}$ +17.4° (*c* 0.5, MeOH); EI-MS *m/z* 279 $(M^+ - H_2O)$; UV λ_{max}^{MeOH} nm (ε) 260 (28,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1720, 1690, 1650, 1630; ^1H NMR (Table 1); ^{13}C NMR (Table 2).

Anal Calcd for $C_{18}H_{27}NO_4 \cdot H_2O$: C 60.94, H 9.27, N 4.44.
Found: C 61.34, H 9.03, N 4.20.

The acidic aqueous solution of above enzymatic degradation was concentrated to dryness. The residue (36 g) was dissolved in 50 ml of water, adjusted to pH 9.0 and applied on a column of reversed phase silica gel (1.6 liters) which was developed with water. The fractions containing compound **XI** were detected by TLC, pooled and concentrated *in vacuo*. The residue was chromatographed on Sephadex LH-20 (250 ml) with 50% aqueous MeOH and then on reversed phase silica gel (250 ml) with acidic water (pH 3.0) to afford pure **XI** hydrochloride, 747 mg (yield 35%). MP 190°C (dec); $[\alpha]_D^{26}$ −113° (*c* 0.5, H_2O); EI-MS *m/z* 241 (M^+); IR ν_{max}^{KBr} cm^{-1} 3400, 1660, 1620, 1520; ^1H NMR (Table 1); ^{13}C NMR (Table 2).

Anal Calcd for $C_{11}H_{19}N_3O_3 \cdot HCl \cdot H_2O$: C 44.67, H 7.50, N 14.21, Cl 11.99.
Found: C 45.04, H 7.82, N 13.81, Cl 12.55.

Acetylation of **XI** (10 mg) by the conventional way (Ac_2O - MeOH) gave mono-*N*-acetyl-**XI** (7 mg): EI-MS *m/z* 283 (M^+); IR ν_{max}^{KBr} cm^{-1} 3400, 1650, 1630, 1540; ^1H NMR (80 MHz, DMSO-d_6) δ 1.23 (3H, d, *J*=7.5 Hz), 1.35~1.6 (4H, m), 1.81 (3H, s), 3.00 (2H, m), 3.52 (1H, m), 4.31 (2H, m), 4.66

(1H, m), 6.11 (1H, d, J=16.3 Hz), 6.46 (1H, dd, J=6.2 and 16.3 Hz), 7.34 (1H, t), 7.98 (1H, d, J=8.0 Hz), 8.57 (1H, d, J=7.2 Hz).

Chemical Synthesis of Ia

(1) Preparation by the Coupling X and XI

A mixture of X (15 mg), DCC (10 mg) and HOBT (8 mg) in 2 ml of DMF was stirred for 2 hours at room temperature. XI (10 mg) was then added to the mixture and stirring continued overnight. The solution was concentrated to a residue which was chromatographed on reversed phase silica gel (40 ml) with 80% MeOH elution. The bioactive fractions were evaporated and the residue was purified by preparative HPLC (column: SSC-ODS-842, mobile phase: 90% aqueous MeOH). Evaporation of the appropriate fractions gave Ia (7.4 mg, yield 28%) which was identical in all respects with the natural antibiotic. TLC (silanized, EtOH - H_2O, 55: 45) Rf 0.45. HPLC (Lichrosorb RP-18, EtOH - H_2O, 65: 35) retention time (Rt): 6.43 minutes.

(2) Total Synthesis

4(*S*)-Chloroacetylamino-2(*E*)-pentenoic Acid (Chloroacetyl-VI)

To a suspension of 4(*S*)-amino-2(*E*)-pentenoic acid (VI, 115 mg, 1 mM) in DMF (3 ml) was added *N*,*O*-bis(trimethylsilyl)acetamide (406 mg, 2 mM) and the mixture was stirred for 0.5 hour at room temperature. Chloroacetyl chloride (113 mg, 1 mM) was added to the solution at 5°C and the mixture was stirred overnight. After dilution with H_2O (20 ml), the mixture was extracted three times with EtOAc (20 ml each). The combined extracts were concentrated to an oil which was triturated with a mixture of EtOAc (10 ml) and *n*-hexane (20 ml). The resulting solid was crystallized from the same solvent system to give 120 mg (63%) of chloroacetyl-VI as colorless needles: MP 126~127°C; IR ν_{max}^{KBr} cm^{-1} 3250, 1710, 1650, 1540.

Anal Calcd for $C_7H_{10}NO_3Cl$: C 43.88, H 5.26, N 7.31, Cl 18.50.
Found: C 43.90, H 5.08, N 7.29, Cl 18.41.

$^{\varepsilon}$*N*-[4(*S*)-Chloroacetylamino-2(*E*)-pentenoyl]-*erythro*-4-hydroxy-L-lysine (XII)

A mixture of chloroacetyl-VI (110 mg, 0.57 mM), DCC (127 mg, 0.62 mM) and HOBT (95 mg, 0.62 mM) in THF (3 ml) was stirred overnight at room temperature. After removing the precipitate by filtration, the filtrate was concentrated and the residue was triturated with *n*-hexane (10 ml) to yield white precipitate of active ester. The solid was added to 50% aqueous DMF (3 ml) solution of *erythro*-4-hydroxy-L-lysine hydrochloride (VII, 113 mg, 0.57 mM) and Et_3N (0.22 ml, 1.7 mM) at room temperature and the mixture was stirred overnight. The mixture was concentrated and residue was chromatographed on a column of Sephadex LH-20 (140 ml). Upon developing with 50% aqueous MeOH, the eluate was examined by TLC (BuOH - AcOH - H_2O, 3 : 1 : 1, ninhydrin detection). The fractions containing the major product were concentrated to a residue which was further purified by a reversed phase silica gel column (20 ml) with 10% aqueous MeOH elution. Evaporation of appropriate fractions afforded 76 mg (39%) of XII as white amorphous solid: MP >189°C (dec); EI-MS m/z 335 (M^+): IR ν_{max}^{KBr} cm^{-1} 3275, 1660, 1625, 1550; ^{1}H NMR (360 MHz, DMSO-d_6) δ 1.19 (3H, d, J=6.3 Hz), 1.5 (3H, m), 1.85 (1H, m), 3.17 (2H, m), 3.72 (1H, m), 4.05 (2H, s), 4.46 (1H, m), 5.92 (1H, dd, J=16.1 and 1.5 Hz), 6.52 (1H, dd, J=16.1 and 6.3 Hz), 7.60 (1H, br s), 8.05 (1H, dd, J=3.1 and 1.5 Hz), 8.42 (1H, d, J=7.2 Hz).

$^{\varepsilon}$*N*-[4(*S*)-Chloroacetylamino-2(*E*)-pentenoyl]-$^{\alpha}$*N*-[*N*-(*tert*-butoxycarbonyl)-L-threonyl]-*erythro*-4-hydroxy-L-lysine-γ-lactone (XIII)

A mixture of *N*-(*tert*-butoxycarbonyl)-L-threonine (46 mg, 0.21 mM), DCC (44 mg, 0.21 mM) and HOBT (32 mg, 0.21 mM) in THF (3 ml) was stirred for 1 hour at room temperature. After removing precipitate by filtration, the filtrate was evaporated to an oil which was dissolved in DMF (0.1 ml) and added to a mixture of XII (70 mg, 0.21 mM), Et_3N (55 μl, 0.42 mM) in 50% aqueous DMF (0.5 ml) with vigorous stirring. The resulting solution was diluted with H_2O (5 ml) and washed with ether (5 ml). The aqueous layer was acidified to pH 2, washed with EtOAc (2×5 ml) and then extracted with BuOH (3×5 ml). The extracts were concentrated and the residue was chromatographed

on a reversed phase silica gel (20 ml) column with a stepwise elution of 10, 30 and 50% aqueous MeOH. The eluate containing **XIII** was combined, evaporated and lyophilized to give colorless amorphous powder of **XIII** (93 mg, 83%): MP >92° (dec); SI-MS *m/z* 541 $(M+Na)^+$; IR ν_{max}^{KBr} cm^{-1} 3300, 1780, 1705, 1670, 1540; ^1H NMR (80 MHz, DMSO-d_6) δ 1.14 (3H, d, J=7.8 Hz), 1.17 (3H, d, J=5.5 Hz), 1.4 (9H, s), 1.75 (2H, m), 3.82 (2H, m), 4.03 (2H, s), 4.5 (4H, m, changed to 2H, m, by D_2O addition), 5.87 (2H, d, J=16.3 Hz), 6.5 (1H, dd, J=5.5 and 16.3 Hz), 8.05 (1H, br s, lost with D_2O), 8.36 (1H, d, J=6.0 Hz, lost with D_2O).

$^{\varepsilon}$*N*-[4(*S*)-Chloroacetylamino-2(*E*)-pentenoyl]-$^{\alpha}$*N*-[*N*-2(*E*),4(*E*)-dodecadienoyl-L-threonyl]-*erythro*-4-hydroxy-L-lysine (XIV)

A mixture of **XIII** (87 mg, 0.16 mM) and formic acid (1.5 ml) was stirred at room temperature for 1 hour. The solution was concentrated, added with H_2O (1.5 ml) and Et_3N (25 μl), and then filtered. The filtrate was chromatographed on a Sephadex LH-20 column (140 ml) with 50% aqueous MeOH elution. The ninhydrin positive fractions were evaporated *in vacuo* to yield highly hygroscopic solid of BOC-free **XIII** (64 mg). 2(*E*),4(*E*)-Dodecadienoic acid (**VIII**, 20 mg, 0.1 mM), was converted to the active ester by reaction with DCC (21 mg, 0.1 mM) and HOBT (15 mg, 0.1 mM) in THF (1 ml). The active ester in DMF (1 ml) was added to a solution of the above deblocked **XIII** (58 mg) in H_2O (1.5 ml) at room temperature. The mixture was stirred for 3 hours and then diluted with H_2O (3 ml), adjusted to pH 2, washed with ether and extracted with BuOH (2×4 ml). The combined extracts were evaporated to a residue which was applied on a column of Sephadex LH-20 (140 ml). Upon elution with 50% aqueous MeOH, the appropriate fractions were pooled, evaporated and lyophilized to give colorless amorphous powder of **XIV** (45 mg, 50% yield from **XIII**): MP >96°C (dec); SI-MS *m/z* 637 $(M+Na)^+$, 615 $(M+H)^+$; UV λ_{max}^{MeOH} nm (ε) 260 (34,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1710, 1660, 1620, 1540; ^1H NMR (80 MHz, DMSO-d_6) δ 0.86 (3H, t, J=6.2 Hz), 1.05 (3H, d, J=7.8 Hz), 1.2~1.8 (17H, m), 2.12 (2H, m), 3.16 (2H, m), 3.9 (1H, m), 4.05 (2H, s), 4.2 (3H, m), 5.86 (1H, d, J=15.8 Hz), 6.10 (3H, m), 6.52 (1H, dd, J=5.1 and 15.8 Hz), 6.98 (1H, dd, J=8.1 and 15.8 Hz), 7.91 (3H, m, lost with D_2O), 8.36 (1H, d, J=8.1 Hz, lost with D_2O).

$^{\varepsilon}$*N*-[4(*S*)-Amino-2(*E*)-pentenoyl]-$^{\alpha}$*N*-[*N*-2(*E*),4(*E*)-dodecadienoyl-L-threonyl]-*erythro*-4-hydroxy-L-lysine (IX)

A mixture of **XIV** (39 mg, 0.06 mM), $NaHCO_3$ (11 mg, 0.13 mM) and thiourea (46 mg, 0.6 mM) in 50% aqueous THF (2 ml) was stirred overnight at room temperature. The mixture was diluted with H_2O (5 ml), adjusted to pH 7 and extracted with BuOH (2×5 ml). The extracts were evaporated to a residue which was chromatographed on reversed phase silica gel (40 ml) with 50 and 70% aqueous MeOH elution. Upon monitoring by ninhydrin test, the ninhydrin-positive fractions were pooled concentrated to give white solid of **IX** (17 mg, 50%). MP 157°C (dec). The physico-chemical properties of the synthesized **IX** was identical with those of **IX** obtained by mild hydrolysis of **Ia**.

Cyclization of IX to Ia

IX (13 mg, 0.024 mM) was stirred with HOBT (3.7 mg, 0.024 mM) and DCC (5 mg, 0.024 mM) in DMF (26 ml) for 18 hours at room temperature. The mixture was evaporated to a residue which was dissolved in H_2O (5 ml) and extracted with two 5 ml-portions of BuOH. The extracts were, after concentration, chromatographed on a reversed phase silica gel column (20 ml) with MeOH - H_2O mixture (3 : 2 to 7 : 3). The eluate was examined by HPLC (Lichrosorb RP-18, MeOH - H_2O, 8 : 2, Rt: 9.8 minutes). The appropriate fractions were concentrated and the residue was crystallized from aqueous MeOH to give 0.3 mg (2.3%) of **Ia**. The identity of the synthetic **Ia** with the natural glidobactin A was confirmed by a direct comparison of their biological properties, spectral data and HPLC.

Ozonolysis of Glidobactin B (Ib)

To a solution of **Ib** (40 mg) in MeOH (10 ml) was introduced ozone at −70°C until the solution became blue. The solution was warmed up to room temperature, concentrated and diluted with water (4 ml). The aqueous solution was refluxed for 20 minutes with zinc dust (200 mg) and then steam-distilled. Treatment of the distillate with 2,4-dinitrophenylhydrazine (106 mg) and conc H_2SO_4

(0.54 ml) yielded yellow precipitate (9.5 mg), which was purified by preparative TLC (benzene - EtOAc, 20 : 1). The yellow major band (Rf 0.73) was collected and extracted with benzene - EtOAc mixture (1 : 1) to give pale yellow amorphous powder (1 mg) which was identified as *n*-hexanal 2,4-dinitrophenylhydrazone: EI-MS *m/z* 280 (M^+); ^{1}H NMR (80 MHz, $CDCl_3$) δ 0.94 (3H, t-like), 1.2~1.7 (6H, m), 2.36 (2H, dt, J=5.2 and 7.5 Hz), 7.49 (1H, t, J=7.5 Hz), 7.89 (1H, d, J=10.0 Hz), 8.27 (1H, dd, J=1.5 and 10.0 Hz), 9.06 (1H, d, J=1.5 Hz).

Acknowledgments

The authors wish to thank Prof. M. OHASHI of the University of Electrocommunication for mass spectroscopic analysis and valuable discussions. Thanks are also due to Dr. B. KRISHNAN of Bristol-Myers Co., Wallingford, Connecticut for ^{1}H NMR (360 MHz) analysis and Dr. T. TSUNO and his associates for their contribution to the spectral analyses of the antibiotics.

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BMY-28190, A NOVEL ANTIVIRAL ANTIBIOTIC COMPLEX

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BMY-28190, an antibiotic complex active against herpes simplex virus type 1 (HSV-1) was produced by the cultured broth of *Streptoalloteichus hindustanus* sp. nov., a producing strain of tallysomycins A and B[1]. The antibiotic complex was recovered from the broth with Amberlite IRC-50 resin and separated from the coproduced tallysomycins and nebramycins by a series of chromatographies. BMY-28190 exhibited weak inhibitory activity toward Gram-positive and Gram-negative bacteria and strong inhibitory activity toward HSV-1. Structural studies disclosed that BMY-28190 is a novel complex of γ-poly-D-α,γ-diaminobutyric acids with an average MW of 5,130.

In an earlier paper[1], we reported isolation of new antitumor antibiotics, tallysomycins A and B, and the aminoglycoside antibiotic nebramycin from the cultured broth of *Streptoalloteichus hindustanus* E465-94. In our search for antiviral antibiotics among microbial metabolites, the broth of this strain showed strong activity toward herpes simplex virus type 1 (HSV-1) which was not caused by the tallysomycins or nebramycins. BMY-28190, the active principle was isolated from the broth by a weakly acidic ion exchange resin and purified by column chromatography using a similar type ion exchanger and silica gel. BMY-28190 is a novel complex of γ-homopolymers of D-α,γ-diaminobutyric acid (D-α,γ-DAB) and exhibits strong inhibition against HSV-1. This paper describes isolation, physico-chemical properties, structure and biological activity of BMY-28190.

Production and Isolation

Taxonomic studies on *S. hindustanus* E465-94 (ATCC 31158) have been reported[2]. The strain was cultivated as described[1] and the harvested broth (30 liters, pH 7.5) was centrifuged. The clear supernate was applied on a column of Amberlite IRC-50 (NH_4^+ type, 3 liters) which was developed with water, 0.01 N NH_4OH, 0.25 N NH_4OH and then with 1.0 N HCl successively. The antiviral activity and nebramycins were eluted by 0.25 N NH_4OH and tallysomycins A and B by 1.0 N HCl. The fractions containing BMY-28190 were concentrated *in vacuo*, and the residue was chromatographed on a column of Amberlite CG-50 (NH_4^+ type, 2×64 cm). After washing with water and elution of most of the nebramycins with 0.1 N NH_4OH, the antiviral activity was eluted with 0.4 N NH_4OH. Upon monitoring the eluate by TLC (SiO_2, $CHCl_3$ - MeOH - 28% NH_4OH - H_2O, 1 : 4 : 2 : 1), the fractions containing BMY-28190 were pooled and evaporated *in vacuo* to afford a semi-pure sample (870 mg). This solid was rechromatographed on a Silica gel column (Wako C-200, 1.5×35 cm) with elution by $CHCl_3$ - MeOH - 28% NH_4OH - H_2O (1 : 4 : 2 : 1) mixture first and then of the upper layer of $CHCl_3$ - MeOH - 28% NH_4OH (1 : 1 : 1). The relevant fractions were concentrated, and the concentrate was applied on a column of Sephadex G-25 (1.0×30 cm) for desalting. Elution was carried out with water, and the course of elution was followed by the TLC as described above. Evaporation of the appropriate fractions yielded a white solid of homogeneous BMY-28190 (54 mg).

Physico-chemical Properties

Physico-chemical data for BMY-28190 are summarized in Table 1. BMY-28190 is readily soluble in water and dimethyl sulfoxide, slightly soluble in methanol and ethanol and practically insoluble in other organic solvents. It gave positive response to ninhydrin, Dragendorff, Rydon-Smith reagents but negative to ferric chloride and anthrone reagents. BMY-28190 does not exhibit absorption in the UV and visible region. The IR spectrum (Fig. 1) indicated amino (3400 cm^{-1}) and amide (1640 and 1540 cm^{-1}) functionalities. BMY-28190 is a complex of several components with similar properties, as depicted in the HPLC (Fig. 2).

Structural Studies

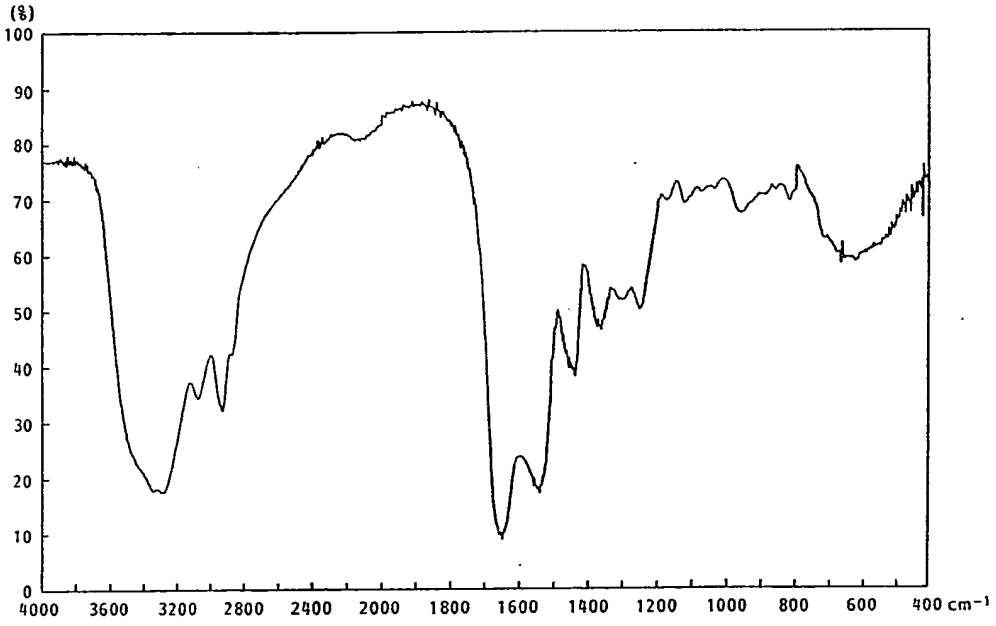
As revealed by HPLC, BMY-28190 is a complex of several components; an average MW of 5,700 was obtained by gel filtration using Sephadex G-50. BMY-28190 was hydrolyzed with 6 N HCl under reflux for 18 hours. The hydrolysate contained only one ninhydrin-positive substance whose be-

Table 1. Physico-chemical properties of BMY-28190.

Nature:	White amorphous solid
MP:	165~167°C
$[\alpha]_D^{25}$ (*c* 1.0, H_2O):	−19°
Microanalysis:	
Calcd for $C_4H_8N_2O \cdot \frac{1}{3}H_2CO_3$:	
	C 43.08, H 7.23, N 23.18.
Found:	C 43.80, H 7.84, N 23.00.
MW:	5,700 (Sephadex G-50 gel filtration chromatography), 5,100~5,200 (DNP method)[7]
TLC, SiO_2*:	Rf 0.02 ($CHCl_3$ - MeOH - 28% NH_4OH - H_2O, 1:4:2:1), Rf 0.72 ($CHCl_3$ - MeOH - 28% NH_4OH, 1:1:1, upper layer)

* Silica gel (Kiesegel $60F_{254}$, Merck). Detection by I_2 vapour and ninhydrin reagent.

Fig. 1. IR spectrum of BMY-28190 (KBr).



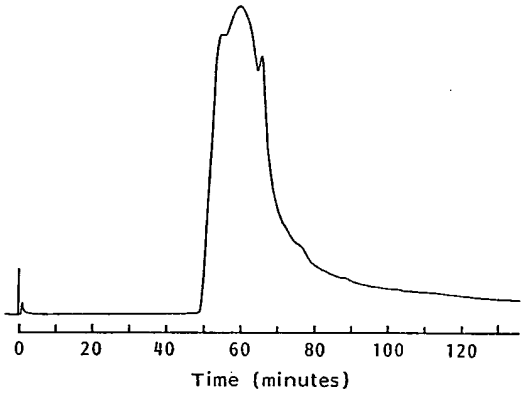
havior in TLC a
was isolated from
less needles by
ethanol. Its spect
($[\alpha]_D^{26.5}$ −20°)[3] c
DAB.

The ^1H NMR
bled that of DAB
signals centered at
Upon acidification,
underwent down-f
appeared as a trip
^1H NMR, the ^{13}C
four carbon signal
and 177.1 ppm (s
those of DAB.
the higher field m

havior in TLC and amino acid analysis was identical with that of α,γ-DAB. This substance was isolated from the hydrolysate by Amberlite CG-50 chromatography and obtained as colorless needles by crystallization from aqueous ethanol. Its spectral data and optical rotation ($[\alpha]_D^{26.5}$ $-20°$)[3] confirmed that it was D-α,γ-DAB.

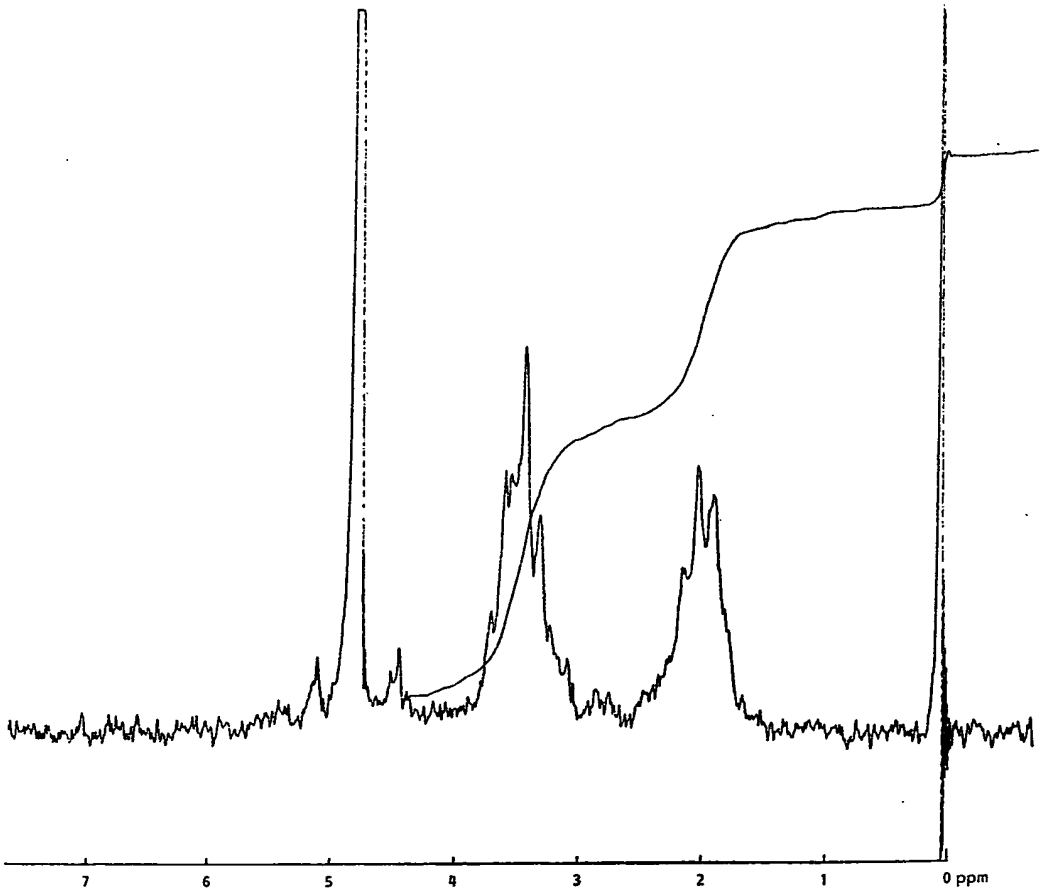
The ^{1}H NMR of BMY-28190 (Fig. 3) resembled that of DAB exhibiting only two multiplet signals centered at 2.00 (2H) and 3.43 ppm (3H). Upon acidification, one proton of the latter signal underwent down-field shift (Δ 0.48 ppm) and appeared as a triplet. In accordance with the ^{1}H NMR, the ^{13}C NMR of BMY-28190 showed four carbon signals at 34.2 (t), 36.7 (t), 53.3 (d) and 177.1 ppm (s) which corresponded well to those of DAB. When determined at pD 2.0, the higher field methylene and carbonyl carbons

Fig. 2. HPLC of BMY-28190.



Tosoh Co. TSK Gel 3000 SW (7.5×600 mm). 1/15 M phosphate buffer, pH 7.0. 0.7 ml/minute, UV at 210 nm.

Fig. 3. ^{1}H NMR spectrum of BMY-28190 (60 MHz in D_2O).



demonstrated protonation shifts of 2.9 and 6.7 ppm, respectively. These spectral data coupled with the hydrolysis result provided convincing evidence that BMY-28190 is γ-homopolymer of D-α,γ-DAB.

For determination of the polymerization rate of DAB, BMY-28190 was reacted with 2,4-dinitrofluorobenzene in aqueous ethanol. The resulting 2,4-dinitrophenyl (DNP)-BMY-28190 was hydrolyzed with 7.1 N H_2SO_4 for 10 hours, and two DNP-DABs produced were separated and purified by preparative TLC. The major one (mp 204~205°C) and the minor one (mp 123~124°C) were identified as α-DNP-DAB[4,5] and α,γ-di-DNP-DAB[5,6], respectively, by comparison with authentic samples. α,γ-Di-DNP-DAB should have been derived from the *N*-terminal and α-DNP-DAB from the other peptide portion of BMY-28190 confirming the assigned linear γ-homopolymer structure of the antibiotic. 2,4-Dinitrophenyllysine has been reported to partly decompose during acid hydrolysis[7,8]. Under the hydrolytic condition used for DNP-BMY-28190, 26.9% of α,γ-di-DNP-DAB and 47.0% of α-DNP-DAB were found to decompose. Taking into consideration these decomposition rates, the ratio of α,γ-di-DNP-DAB and α-DNP-DAB produced in the hydrolysis of DNP-BMY-28190 was calculated as 1 : 50.3 indicating an average MW of 5,130 (51~52 homopolymer) for the antibiotic.

$$NH_2CH_2CH_2\underset{\underset{NH_2}{|}}{CH}CO(NHCH_2CH_2\underset{\underset{NH_2}{|}}{CH}CO)n\text{-}NHCH_2CH_2\underset{\underset{NH_2}{|}}{CH}COOH$$

BMY-28190

n=49~50 (average)

Biological Activity

Antiviral Activity

Antiviral activity of BMY-28190 was assessed by the plaque reduction assay and dye-uptake assay[9] using the HSV-1-vero cell system. ε-Poly-L-lysine[10], a structurally related agent produced by *Streptomyces* No. 346 and two types of synthetic α-poly-L-lysine (MW 3,500 and 25,000) were tested comparatively as reference compounds. The results are shown in Table 2 together with their cytotoxicity against host cells. By plaque reduction assay, BMY-28190 was the most potent among the compounds tested, showing an ID_{50} of 0.84 μg/ml. ε-Poly-L-lysine and higher molecular α-poly-L-lysine were slightly less active than BMY-28190, and lower molecular α-poly-L-lysine was the least active. BMY-28190 and ε-poly-L-lysine were comparably active by the dye-uptake

Table 2. Activity against herpes simplex virus type 1.

	Plaque reduction assay		Dye-uptake assay	
	Activity vs. HSV-1 (ID_{50}: μg/ml)	Cytotoxicity vs. vero cell ($TCID_{50}$: μg/ml)	Activity vs. HSV-1 (ID_{50}: μg/ml)	Cytotoxicity vs. vero cell ($TCID_{50}$: μg/ml)
BMY-28190	0.84	170	2.8	540
ε-Poly-L-lysine	2.6	70	2.7	>100
α-Poly-L-lysine (MW 3,500)	42	>400	100	>100
α-Poly-L-lysine (MW 25,000)	1.8	25	>20	20

Cells: Vero cells. Medium: EAGLE MEM containing 5% fetal bovine serum.

Table 3. Antibacterial activity by the broth dilution method.

Test organisms	MIC (μg/ml)		
	BMY-28190	ε-Poly-L-lysine	α-Poly-L-lysine
Staphylococcus aureus Smith	6.3	6.3	12.5
S. aureus BX-1633-2	6.3	6.3	25
Streptococcus faecalis A9612	>100	50	>100
Escherichia coli Juhl	6.3	6.3	12.5
Klebsiella pneumoniae A9977	3.1	6.3	12.5
Proteus mirabilis A9554	25	6.3	50
Serratia marcescens A20019	>100	25	>100
Enterobacter cloacae A9656	100	25	>100
Haemophilus influenzae A2241	100	12.5	100
Pseudomonas aeruginosa A9930	100	12.5	>100

assay, while the two α-poly-L-lysines were nearly inactive. BMY-28190 showed relatively weak cytotoxicity and had the best selectivity in these assay systems.

Table 4. Antiviral activity of partial hydrolysates of BMY-28190 by dye-uptake assay.

Peptide fragment No.	Number of DAB	Anti-HSV-1 activity ID_{50} (μg/ml)
D-α,γ-DAB	1	>660
1	3~4	>660
2	6~7	>660
3	8~9	>660
4	9~10	>660
5	12~13	>660
6	15~16	220
7	16~17	38
8	21~22	23
BMY-28190	51~52	2.8

Antibacterial Activity

The *in vitro* antibacterial activity of BMY-28190 was determined by the 2-fold serial broth dilution method in nutrient broth using ε-poly-L-lysine and α-poly-L-lysine (MW 3,500) as reference compounds. As shown in Table 3, these three compounds exhibited moderate inhibitory activity against Gram-positive and Gram-negative bacteria with a similar antibacterial spectrum. In terms of MICs, BMY-28190 was comparably active to ε-poly-L-lysine against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* but less active than ε-poly-L-lysine against other organisms. α-Poly-L-lysine was the least active against all test strains.

Acute Toxicity

The LD_{50} of BMY-28190 was 22.6 mg/kg following intramuscular administration to male *dd*Y mice.

Partial Acid Hydrolysis of BMY-28190 and Anti-HSV-1 Activity of the Products

In order to examine the relationship between peptide chain length and antiviral activity, partial acid hydrolysis of BMY-28190 was carried out by heating with 6 N HCl at 100°C for 1 hour. The hydrolysate was chromatographed on a CM-cellulose column to yield 8 peptide fragments. Numbers of D-α,γ-DAB in each peptide were determined by the DNP method as described before, and antiviral activity against HSV-1 was assessed by dye-uptake assay (Table 4). Small peptides with less than 12 D-α,γ-DAB residues were practically inactive against the viruses, while peptide fragments 6, 7 and 8 having more than 15 amino acids showed weak antiviral activity. Potency increased with the length of the peptide chain. BMY-28190 was more active than these peptides.

Discussion

BMY-28190, a complex of novel antiviral antibiotics, was produced by *S. hindustanus* E465-94 together with tallysomycins and nebramycins. It is active against herpes simplex virus type 1 both by plaque reduction assay and dye-uptake assay and shows moderate antibacterial activity against certain Gram-positive and Gram-negative bacteria. Structural studies revealed that BMY-28190 is a mixture of linear γ-homopolymers of D-α,γ-DAB with an average MW of 5,130.

SHIMA and SAKAI reported the isolation of ε-poly-L-lysine from the fermentation broth of *Streptomyces albulus*[10], and in their recent papers, they demonstrated that the compounds had bacteriophage inactivation activity[11] and antimicrobial activity[12]. ε-Poly-L-lysine was demonstrated to show antiviral activity against HSV-1 in our test system. BMY-28190 is the second example of bioactive homopolymers of amino acids obtained from natural origins. It is worthy to note that BMY-28190 is a larger polymer ($n=51\sim52$) than ε-poly-L-lysine ($n=25\sim30$) and is composed of D-amino acid in contrast to L-amino acid polymers of the latter. In addition, it is interesting that homopolypeptides consisting of more than 15 D-α,γ-DAB residues were active against HSV-1, while smaller polymers were inactive as revealed by partial hydrolysis experiments.

Acknowledgments

We want to thank late Prof. H. SAKAI of the University of Osaka Prefecture for providing us the sample of ε-poly-L-lysine. Thanks are also due to the members of Microbiology, Fermentation and Analytical groups for their excellent technical assistance.

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Notes

ESPERAMICINS, A NOVEL CLASS OF POTENT ANTITUMOR ANTIBIOTICS I. PHYSICO-CHEMICAL DATA AND PARTIAL STRUCTURE

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Recently we have isolated several members of a family of exquisitely potent antitumor antibiotics from *Actinomadura verrucosospora*, strain H964-62 (BBM-1675, ATCC 39334).[1] The producing organism was collected at Pto Esperanza, Misiones, Argentina, consequently we have named these compounds esperamicins. The recent appearance of several patents and papers describing what appear to be related compounds prompts us to report the physicochemical properties of esperamicins A_1 (1), A_2 (2), and A_{1b} (3) as well as the partial structure of esperamicins A_1 and A_2.[2,3]

The esperamicins have been isolated from the fermentation beers *via* broth extraction with butanol, concentration, and precipitation by *n*-hexane.† The solids thus obtained were chromatographed on Sephadex LH-20, silica gel and finally on reverse phase HPLC supports. To date we have isolated esperamicins A_1, A_{1b}, A_3, A_4, A_4, B_1 and B_2, compound A_{1b} and the latter four components being minor ones.[1)††]

† A manuscript detailing the taxonomy, fermentation, and biological activities of the esperamicins is in preparation, T. MIYAKI, K. TOMITA, H. KAMEI *et al.*

†† Full details of the isolation procedures will be forthcoming, M. KONISHI, H. OHKUMA, J. A. MATSON & D. E. NETTLETON.

Esperamicin A_1 was isolated as white to pale yellow crystals, mp 156~158°C (dec), $[\alpha]_D^{25}$ −207° (*c* 0.0351, $CHCl_3$) and $[\alpha]_D^{25}$ −191° (*c* 0.5, $CHCl_3$). The IR spectrum of **1** had bands characteristic of hydroxyl groups, amide, ester and α,β-unsaturated ketone (IR bands at 3440, 3360, 2960, 2920, 1715, 1668, 1608, 1592, 1520, 1446, 1405, 1380, 1308, 1250, 1210, 1150, 1110, 1070, 1015, 985 cm^{-1}). The UV spectrum of **1** in MeOH showed bands at λ_{max} (nm) 320 (*a* 12.4), 280 (sh), 253 (*a* 25.1), 210 (*a* 25.5). No significant shifts were observed upon addition of either acid or base. The elemental formula of esperamicin A_1 was determined to be $C_{55}H_{84}N_4O_{22}S_3$ using elemental analysis and FAB high resolution mass spectroscopy. The elemental analysis gave values of C 52.17%, H 6.15%, N 4.63%, S 9.09% and O 27.96% (by difference). The ^1H NMR spectrum of **1** at 360 MHz in $CDCl_3$ exhibited resonances at δ 11.75 (1H, s); 8.55 (1H, s); 7.45 (1H, s); 6.61 (1H, m); 6.23 (1H, br s); 6.17 (1H, br s); 5.93 (1H, d, J=9.3 Hz); 5.82 (1H, d, J=9.3 Hz); 5.70 (1H, br s); 5.49 (1H, m); 5.45 (1H, d, J=2.3 Hz); 5.38 (1H, br s); 4.95 (1H, d, J=10.2 Hz); 4.64 (2H, m); 4.54 (1H, d, J=2.3 Hz); 4.20 (1H, s); 4.15~3.35 (26~28H including 4.10 (1H, m), 4.02 (1H, br s), 3.95 (3H, s), 3.85 (3H, s), 3.79 (3H, s), 3.46 (1H, m), 3.40 (3H, s)); 2.82~2.70 (3H, br m); 2.50 (3H, s); 2.47 (1H, m); 2.38~2.22 (5H); 2.12 (1H, m); 2.11 (3H, s); 1.60~1.05 (22H including 1.39 (3H, d, J=6.3 Hz), 1.31 (3H, d, J=6.3 Hz), 1.29 (3H, d, J=6.3 Hz) 1.08 (6H)). The ^{13}C NMR spectrum of **1** is recorded in Table 1 together with those of **2**, **4**, and **5** for comparison purposes.

Esperamicin A_2 (**2**) was isolated as white crystals, mp 147~149°C, $[\alpha]_D^{25}$ −179.4° (*c* 0.5, $CHCl_3$). The IR and UV spectra of **2** were very similar to those of **1**. The elemental formula and molecular weight of **2** were shown to be identical to that of **1** by FAB high resolution mass spectroscopy. The elemental analysis gave values of C 52.71%, H 5.94%, N 3.94%, S 9.39%, O 28.02% (by difference). The ^1H NMR spectrum of **2** at 360 MHz in $CDCl_3$ exhibited some differences from that of **1**. The resonances were observed at δ 11.91 (1H, s); 8.62 (1H, s); 7.58 (1H, s); 6.56 (1H, m); 6.22

Table 1. ^{13}C NMR spectra of compounds 1, 2, 4 and 5.[a]

Carbon	[b]	1	2	4	5	Carbon	[b]	1	2	4	5
1	q	13.7	13.7			29	d	76.6	76.9		
2	q	16.6	16.9	16.6	16.9	30	u	77.1	77.7		
3	q	17.5	17.5			31	d	77.3	78.1		
4	q	19.8	19.8			32	s	83.4	83.3		
5	q	22.2	22.3			33	d	86.6	86.2		
6	q	22.6	22.6			34	s	88.4	88.4		
7	q	23.4	23.4			35	t	90.5	90.4	90.6	90.4
8	t	29.0	33.1	29.0	33.3	36	d	97.2	97.2		
9	t	34.0	34.0			37	s	98.3	98.3		
10	t	35.1	35.1			38	d	99.0	99.1	99.0	98.8
11	t	39.5	39.3			39	d	99.5	99.1		
12	d	47.2	47.6			40	d	99.5	99.5		
13	q	52.5	52.6			41	d	103.7	103.8	103.8	103.9
14	u	55.6	55.7			42	s	107.1	107.6	107.1	107.1
15	q	56.0	56.0	56.0	56.1	43	d	112.5	112.4	112.6	112.7
16	q	56.0	56.1	56.0	56.1	44	d	123.1	123.2		
17	q	56.0	56.1	56.0	56.1	45	d	124.9	124.8		
18	d	57.1	57.6			46	d	130.1	129.9		
19	t	62.3	62.4			47	s	131[c]	131[c]		
20	d	64.5	64.5			48	s	136.7	137.3	136.8	137.5
21	d	66.7	65.9	66.6	65.2	49	s	144.0	144.1	144.1	144.2
22	t	68.2	68.2			50	s	147[c]	147[c]		
23	d	68.8	73.6	68.8	73.4	51	s	153.8	154.2	153.9	154.4
24	d	69.2	69.2			52	s	154.4	154.5	154.4	154.5
25	t	69.6	69.7			53	s	160.7	160.9	160.9	160.9
26	d	70.2	64.9	70.2	64.7	54	s	166.7	167.9	166.5	168.0
27	d	71.8	71.9			55	s	191.8	192.0		
28	d	76.0	75.8								

[a] Recorded at 90 MHz in $CDCl_3$ on a Bruker WM360.
[b] Multiplicity q=quartet, t=triplet, d=doublet, s=singlet, u=uncertain.
[c] Broad diffuse signals.

(1H, s); 6.15 (1H, br s); 5.91 (1H, d, J=9.6 Hz); 5.83 (1H, d, J=9.6 Hz); 5.70 (1H, m); 5.45 (1H, d, J=2.2 Hz); 5.44 (1H, s); 5.34 (1H, br s); 4.95 (1H, d, J=10.2 Hz); 4.75 (1H, m); 4.65 (1H, d, J=6.8 Hz); 4.54 (1H, d, J=2.2 Hz); 4.47 (1H, m); 4.18 (1H, s); 4.10 (1H, br s); 4.05~3.50 (20~24H, including 3.96 (3H, s), 3.87 (3H, s), 3.77 (3H, s)); 3.46 (1H, m); 3.39 (3H, s); 2.79 (1H, m); 2.73 (2H, m); 2.50 (3H, s); 2.50 (1H, m); 2.38~2.22 (3H, m); 2.14 (1H, m); 2.10 (3H, s); 1.98 (2H, m); 1.65~1.45 (6~8H); 1.38 (3H, d, J=6.0 Hz); 1.34 (3H, d, J=6.0 Hz); 1.22 (3H, d, J=6.8 Hz); 1.10 (6H).

Esperamicin A_{1b} (3) was isolated as a minor congener of the isolation of 1. Examination of its physico-chemical properties led to the conclusion that it is identical with WS 6049A discovered earlier by KIYOTO *et al.*[2~4] The ^{13}C NMR spectrum recorded for 3 was identical with that reported for WS 6049A within experimental error. One discrepancy between our data and that of KIYOTO's group is the molecular weight. We have determined the molecular weight of 3, using FAB high resolution mass spectroscopy, to be 1,235.469 corresponding to an [M+H] ion $C_{54}H_{83}N_4O_{22}S_3$. This represents a difference between compounds 1 and 2 and compound 3 of a single methyl function which is in complete accord with the ^{13}C evidence. The Fujisawa group reports an [M+H] ion at 1,311 determined by FABQ-MS. There are several possible reasons for the discrepancy one of which may be a matrix effect described in further detail below.

Mass Spectroscopy

The molecular weights of esperamicins A_1 and A_2 (1 and 2) were determined to be 1,248.456

4	5
90.6	90.4
99.0	98.8
103.8	103.9
107.1	107.1
112.6	112.7
136.8	137.5
144.1	144.2
153.9	154.4
154.4	154.5
160.9	160.9
166.5	168.0

experimental
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be 1,248.456

using high resolution FAB-MS. This value corresponds to an elemental composition of $C_{55}H_{84}N_4O_{22}S_3$ which was determined by an analysis of all the data. Initially the molecular weight determinations were complicated by unexpected matrix effects which lead to incorrect assignments of the [M+H] ion. In order to unambiguously assign the molecular formulas of the esperamicins we have studied the FAB-MS using three matrices, thioglycerol, dithiothreitol/dithioerythritol "magic bullet"* (MB), and glycerol.

The thiol containing matrices yield abundant high mass ions at [M+H] and [M+H+matrix]. The FAB experiments with glycerol did not contain abundant high mass ions. Addition of DMSO to the glycerol matrix facilitated dissolution of **1** while at the same time accelerating its decomposition leading to very complex spectra which changed over time greatly diminishing the value of these experiments.**

In the thiol containing matrices, **1** and **2** yield a protonated molecular ion [M+H] cluster at *m/z* 1,249 daltons and more intense [M+H+matrix] ion clusters at *m/z* 1,357 and *m/z* 1,403 (when thioglycerol and MB matrices are used respectively). The [M+H] ion clusters were confirmed by adding NaCl to the mixtures. This yielded more intense ions at [M+Na] *m/z* 1,271 and [M+Na+matrix]. For spectra taken in thioglycerol with and without addition of NaCl the ratio of the [M+H] to the [M+H+matrix] was 0.20. A single matrix could lead to mistaking the [M+H+matrix] ion cluster for the [M+H] ion.[2-6] However a comparison of the thioglycerol and MB matrix spectra leads to proper assignment of the [M+H] ion. The matrix effect observed for **1** and **2** was also observed for **3** thus implying that this may be a general effect for molecules of this class.

* "Magic bullet" (MB) is a mixture of dithiothreitol: dithioerythritol (3:1) developed by J. CARTER COOK & K. RINEHART.

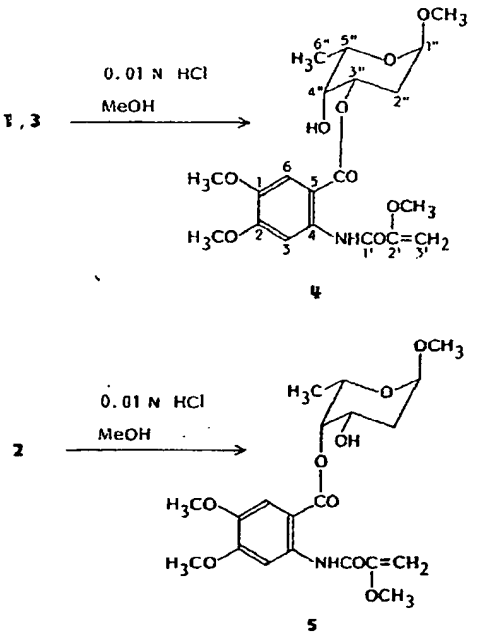
** The esperamicins are extremely reactive molecules. Consequently it is necessary that samples for FAB-MS be run immediately following preparation. The isotope ratios of prominent ion clusters change within five minutes of sample preparation. A matrix effect was observed for DMSO solutions of compound **1**, *i.e.* there was a strong [M+H+DMSO] ion at *m/z* 1,325.

Partial Structure Determination

Examination of the physico-chemical data for the esperamicins lead to the conclusion that these molecules consist of a chromophore conjugated to a number of sugars as shown by the anomeric carbons in the ^{13}C together with the corresponding protons in the ^1H NMR spectra. There is also evidence that there is an α,β-unsaturated ketone present in the molecule (^{13}C NMR signal at 192 ppm). Unfortunately the complexity of the esperamicin structure(s) precluded a complete NMR analysis. Consequently we have carried out a number of degradations of **1**, **2**, and **3** with a view to providing simpler fragments.

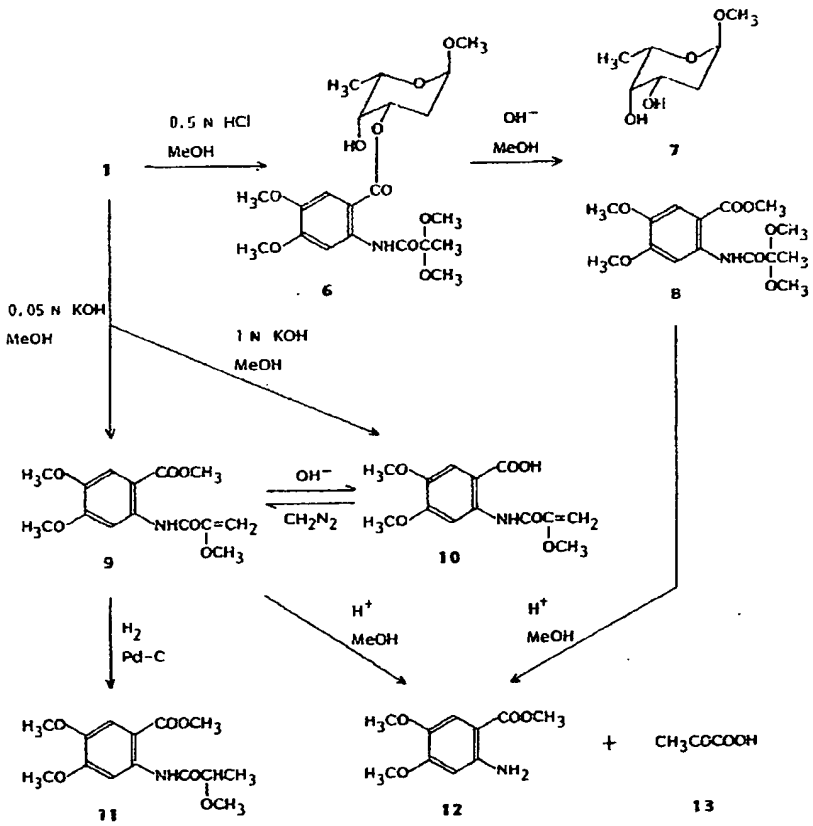
From an analysis of the ^1H and ^{13}C NMR spectra of **1** and **2** together with the mass spectra evidence it was apparent that **1** and **2** were close structural analogs if not isomers of the same basic structure. Methanolysis of **1** using 0.01 N hydrogen chloride (Scheme 1) yielded fragment **4** together with several other products. Compound **4** was also obtained upon methanolysis of **3**. When compound **2** was subjected to these conditions, **5**, a compound closely related to but different from **4**, was obtained. The IR and UV spectra of **4** and **5** were essentially superimpossible. The IR spectrum had absorptions

Scheme 1.



which corresponded to hydroxyl groups, aromatic ester, amide, and enol ethers; 3470, 3250, 2940, 2840, 1690, 1610, 1600, 1530, 1450, 1410, 1370, 1350, 1310, 1255, 1215, 1160, 1125, 1080, 1050, 1005, 990, 945, 920, 880, 860, 780, 750 cm^{-1}. The UV spectrum in MeOH indicated that the chromophore from the esperamicins was present in the fragments, λ_{max} nm, 323 (*a* 27.7), 252 (*a* 63.7), 210 (*a* 19.1) (*c* 0.0204 g/liter). The ^1H NMR spectrum of **4** in $CDCl_3$ at 360 MHz showed resonances at δ 11.75 (1H, bs, NH); 8.49 (1H, s, C3-H); 7.42 (1H, s, C6-H); 5.48 (1H, d, J=2.5 Hz, C3'-H); 4.50 (1H, d, J=2.5 Hz, C3'-H); 5.38 (1H, m, C3''-H); 4.85 (1H, m, C1''-H); 4.03 (1H, dq, C5''-H); 3.93 (3H, s, C1-OCH_3); 3.93 (1H, m, C4''-H); 3.83 (3H, s, C2-OCH_3); 3.74 (3H, s, C2'-OCH_3); 3.34 (3H, s, C1''-OCH_3); 2.21, 1.97 (2H, m, C2''-H_2); 1.29 (3H, d, C6''-CH_3). The ^1H NMR spectrum of **5** in $CDCl_3$ at 360 MHz was similar to that of **4** with the exception of the resonances for C3''-H (at δ 4.31), C4''-H (at δ 5.28), and C5''-H (at δ 4.90) indicating that **4** and **5** differ in the substitution at the C3'' and C4'' hydroxyl groups of a 2-deoxyfucose unit. This was further supported by the ^{13}C NMR spectra (listed in Table 1) of compounds **4** and **5**. The resonances for all 20 carbons in **4** and **5** have been unequivocally assigned* as follows — compound **4**: 16.6 (C6''), 29.0 (C2''), 54.7 (C1''-OCH_3), 56.0 (C1-OCH_3, C2-OCH_3, C2'-OCH_3), 66.6 (C5''), 68.8 (C4''), 70.2 (C3''), 90.6 (C3'), 99.0 (C1''), 103.8 (C3), 107.6 (C5), 112.6 (C6), 137.5 (C4), 144.1 (C1), 153.9 (C2), 154.4 (C2'), 160.9 (C1'), 166.5 (C5-COOR). Compound **5**: 16.9 (C6''), 33.3 (C2''), 54.9 (C1''-OCH_3), 56.1 (C1-OCH_3, C2-OCH_3, C2'-OCH_3), 65.2 (C5''), 73.4 (C4''), 64.7 (C3''), 90.4 (C3'), 98.8 (C1''), 103.9 (C3), 107.1 (C5), 112.7 (C6), 137.5 (C4), 144.2 (C1), 154.4 (C2), 154.5 (C2'), 160.9 (C1'), 168.0 (C5-

Scheme 2.



* The proton and carbon assignments were made by homonuclear and heteronuclear correlation spectroscopy.

COOR). From an analysis of the NMR data it is evident that in compound **4** the 2-deoxyfucose is substituted at C3″ while in **5** it is substituted at C4″. Examination of the ^{13}C NMR spectra of **1** and **2** in comparison with those of **4** and **5** indicates that **1** and **2** are isomers about C3″ and C4″ of the 2 deoxyfucose unit as well.

Further evidence for the structures of **4** and **5** was provided by additional degradative work on compound **1**. (Scheme 2). Methanolysis of **1** under more vigorous conditions (0.5 N HCl) yielded **6**. The NMR spectrum of **6** was similar to that of **4** with the exception that the vinylogous methylene unit of **4** had disappeared to be replaced by a singlet methyl group. In addition there was an additional methoxyl group in **6**. Treatment of **6** with dilute base in MeOH gave the α-methylglycoside of 2-deoxyfucose **7** plus compound **8**. Further methanolysis of **8** (1.5 N HCl) gave **12** identified as methyl 4,5-dimethoxyanthranilate by comparison with an authentic sample. Treatment of the hydrolysate residues from this experiment with 2,4-dinitrophenylhydrazine gave the hydrazone of pyruvic acid. The absolute configuration of methyl α-2-deoxyfucopyranoside (**7**) was determined by application of the CD method to the 3,4-di-*p*-bromobenzoate of **7**. The CD spectrum of this compound exhibited a strong negative first Cotton effect and split CD curve ($\Delta\varepsilon_{233\,nm}$ −20.2, $\Delta\varepsilon_{244\,nm}$ 0, $\Delta\varepsilon_{257\,nm}$ +11.2). This is consistent with 2-deoxy-L-fucose.[7]

Base treatment of **1** with dilute hydroxide (0.05 N KOH) in MeOH gave **9**. When more vigorous treatment was applied (1 N KOH-MeOH) the carboxylic acid **10** corresponding to **9** was obtained. Compound **9** could be converted to **10** *via* base hydrolysis and **10** to **9** *via* treatment with diazomethane. Hydrogenation of **9** gave **11** in which the exomethylene unit of **9** had disappeared. Compound **11** exhibited a new doublet methyl signal and one of the methoxyl groups in **9** had shifted to higher field (δ 3.50) thus confirming in **9** the presence of a $C{=}CH_2$ unit. Hydrolysis of **9** gave **12**.

OCH_3

Discussion

The above data establishes partial structures for esperamicins A_1, A_2 and A_{1b} (1~3) consisting of an aromatic chromophore ester of 2-deoxy-L-fucose as an α-glycoside of the remaining portion of the molecule. In compounds **1** and **3** the C3 hydroxyl function of the sugar is esterified while in **2** the C4 hydroxyl group is substituted. Structural studies on the esperamicins are in progress and their full structures will be the subject of future manuscripts.

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EXHIBIT 0

CHICAMYCIN*, A NEW ANTITUMOR ANTIBIOTIC

II. STRUCTURE DETERMINATION OF CHICAMYCINS A AND B

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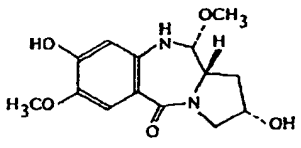
Structures of chicamycins A and B have been determined from a series of chemical degradation studies coupled with spectroscopic analysis. Chicamycin A is 2(*S*),11(*R*),11a(*S*)-1,2,3,10,11,11a-hexahydro-2,8-dihydroxy-7,11-dimethoxy-5*H*-pyrrolo-[2,1-c][1,4]-benzodiazepin-5-one, and chicamycin B is 2(*S*),11a(*S*)-1,2,3,11a-tetrahydro-2,8-dihydroxy-7-methoxy-5*H*-pyrrolo-[2,1-c][1,4]-benzodiazepin-5-one which is the demethanol form of chicamycin A. The structure of chicamycin B is closely related to that of neothramycin, differing only in the position of a hydroxyl substituent on the pyrrolidine ring.

Chicamycin is a new member of the pyrrolobenzodiazepine family of antibiotics elaborated by a strain of *Streptomyces* sp. (J576-99). It was obtained in two active forms, chicamycins A and B, depending upon the isolation procedure used. Both forms of the antibiotic exhibit an antitumor effect on murine leukemia along with weak antimicrobial activity against some Gram-positive bacteria. The taxonomy of strain J576-99, production, isolation and chemical and biological properties of chicamycin have been reported in the preceding paper[1]. This paper presents evidence that chicamycins A and B have the structures shown in Fig. 1.

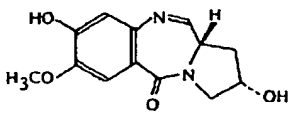
General Structural Characteristics

The UV absorption maxima of chicamycins A (**Ia**) and B (**Ib**) were observed at 223, 237, 262 and 317 nm in methanol which resembled those of neothramycin[2] and tomaymycin[3] but differed from those of sibiromycin[4], anthramycin[5] and mazethramycin[6] (Fig. 2). Thus, the chromophore part of chicamycin was assumed to be similar to that of neothramycin or tomaymycin. The mass spectrum of **Ia** showed the molecular ion peak at *m/z* 294 and the base peak ion at *m/z* 262, corresponding to a loss of a methanol unit from the molecule. The molecular ion of **Ib** appeared at *m/z* 262 and the fragment ions of **Ib** were almost consistent with those of **Ia** suggesting that **Ib** should be a demethanol form of **Ia**. This was evidenced by comparison of their ^{1}H NMR spectra: the ^{1}H NMR spectrum of **Ib** lacked one of the two OCH_3 (δ 3.30 ppm) groups and the NH proton (δ 7.94 ppm) observed with **Ia**, while an ad-

Fig. 1. Structures of chicamycins A and B.



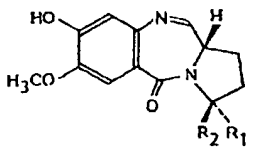
Chicamycin A (**Ia**)



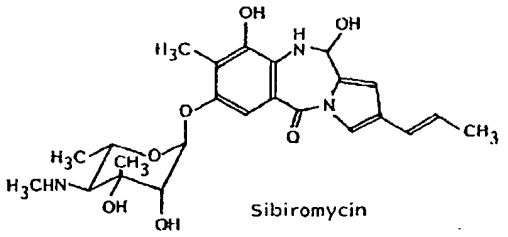
Chicamycin B (**Ib**)

* This antibiotic was originally designated as BBM-2040.

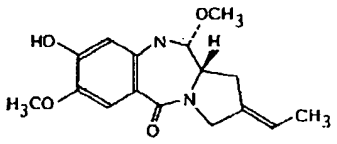
Fig. 2. Structures of pyrrolo-[1,4]-benzodiazepine antibiotics.



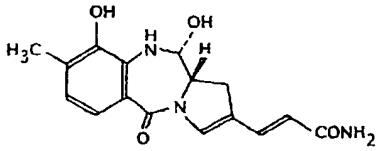
Neothramycin A $R_1 = OH$, $R_2 = H$
Neothramycin B $R_1 = H$, $R_2 = OH$



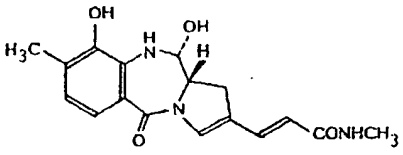
Sibiromycin



Tomaymycin



Anthramycin



Mazethramycin A

ditional double bond proton (δ 8.24 ppm) was present in the spectrum of **Ib**. **Ib** was prepared from **Ia** in a good yield when **Ia** was treated with pyridine at room temperature, while **Ib** was converted to **Ia** by heating with methanol. The present structure study was performed mostly with crystalline chicamycin A (**Ia**).

^{1}H NMR and ^{13}C NMR Spectra of Chicamycin A

The 360 MHz NMR of **Ia** was determined in pyridine-d_5. The observed signals and their assignments are given in Table 1. The signals assignable to two aromatic protons (6.88 and 8.17 ppm), one NH (7.94), one phenolic OH (11.68) and two OCH_3 (3.30 and 3.75) are very similar to the corresponding signals of tomaymycin determined under the same conditions. The proton on the carbinolamine carbon (H_{11}, 4.77) resonated as a doublet which collapsed into a singlet upon irradiation of the NH proton. The lack of coupling between H_{11} and H_{11a} is commonly observed in the anthramycin-tomaymycin group of antibiotics which have 11-*R* and 11a-*S* configurations[11]. Thus it seems that the 1,4-benzodiazepine moiety of chicamycin A is identical with that of tomaymycin. In the ^{1}H NMR spectrum, the alcoholic proton appeared as a doublet at 6.34 ppm. The decoupling experiment revealed that the alcoholic proton coupled with a methine proton at 4.53 ppm (H_2) which, in turn, coupled with the high-field methylene protons (δ 2.39, H_{1A} and 2.57, H_{1B}) and also a proton at 4.14 ppm (H_{3A}). Irradiation of either of the non-equivalent methylene protons (H_{1A} and H_{1B}) converted a triplet proton at 4.08 ppm (H_{11a}) into a doublet, accompanied by a significant change in the coupling pattern of H_2 proton. Therefore, it was concluded that the secondary hydroxyl group of chicamycin A was located at C-2 of the pyrrolidine ring.

The ^{13}C NMR spectrum of chicamycin A supported the structure as determined by the ^{1}H NMR analysis. The presence of 14 carbon signals was indicated by the ^{13}C NMR spectrum as shown in Table

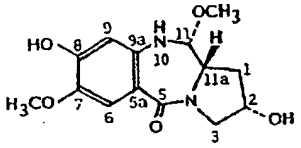
Table 1. ^{1}H NMR (360 MHz) of chicamycin A (in pyridine-d_5).

Chemical shift δ (ppm)	Proton	Coupling multiplicity (J: Hz)	Assignment
2.39	1H	m	H_{1A}
2.57	1H	m	H_{1B}
3.30	3H	s	C_{11}–OCH_3
3.75	3H	s	C_7–OCH_3
4.08	1H	t (8.1)	H_{11a}
4.14	1H	dd (12.0 & 5.8)	H_{3A}
4.48	1H	dd (12.0 & 6.0)	H_{3B}
4.53	1H	m	H_2
4.77	1H	d (J=6.4)	H_{11}
6.34	1H	d (J=7.4)	C_2–OH
6.88	1H	s	H_9
7.94	1H	d (J=6.4)	N_{10}–H
8.17	1H	s	H_3
11.68	1H	s	C_6–OH

Table 2. ^{13}C NMR of chicamycin A (in pyridine-d_5).

Carbon	Chemical shift (δ: ppm)	Multiplicity on off-resonance
1	24.4	t
2	53.3	d
3	41.9	t
5	151.7***	s
5a	126.8*	s
6	90.0	d
7	137.5	s
8	150.1***	s
9	101.6	d
9a	125.4*	s
11	73.4	d
11a	43.4	d
7-OCH_3	41.4**	q
11-OCH_3	38.9**	q

*, **, ***: Assignments may be interchangeable.



2. Although most of the signals appeared at considerably lower field than the corresponding carbons of the related antibiotics determined in deuterodioxane or DMSO-d_6, the carbon signals of chicamycin A were assigned as shown in Table 2 on the basis of off-resonance decoupling and comparison with the literature data of neothramycin[7] and pretomaymycin[8].

Acid Hydrolysis of Chicamycin A (Ia)

In order to confirm the structure assigned by the ^{1}H NMR and to determine the configuration of C_2-OH, cleavage of **Ia** was carried out by two routes. On acetylation in pyridine (Fig. 3), **Ia** afforded the di-*O*-acetyl-demethanol derivative **II**, (M^+ m/z 346), indicating the presence of two acetylable hydroxyl groups in **Ia**. The removal of a methanol unit from **Ia** occurred during acetylation as was indicated by the ^{1}H NMR spectrum. This was verified by the fact that the same acetyl derivative was obtained by acetylation of **Ib**. **II** was treated with *m*-chloroperbenzoic acid at −20°C for 3 hours[9]. After removal of the acidic by-products by washing with alkali, the reaction mixture was chromatographed on silica gel to give the pure oxo-compound **III** (M^+ m/z 362). The ^{1}H NMR spectrum of **III** indicated the absence of N=CH (7.83 ppm, doublet) observed for **II** and the appearance of NH-CO (9.07 ppm, singlet). The IR spectrum in KBr showed a new amide carbonyl band at around 1690~1700 cm^{-1}.

Fig. 3. Acid degradation of chicamycin A.

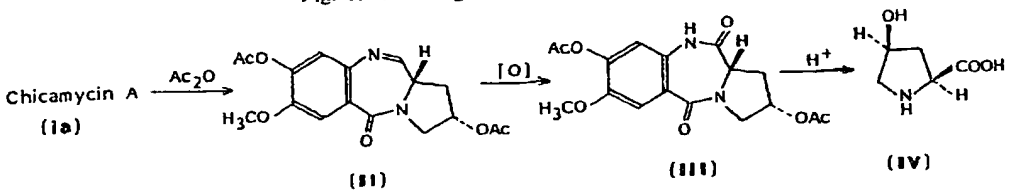


Table 3. Physico-chemical properties of compound IV, *cis*-4-hydroxy-L-proline and *trans*-4-hydroxy-L-proline.

	Compound IV	*cis*-4-OH-L-proline	*trans*-4-OH-L-proline
Mp	258~259°C (dec)	257~258°C (dec)	273~275°C (dec)
$[\alpha]_D^{25}$ (*c* 1.0, H_2O)	−51.5°	−56.5°	−75.3°
TLC Solvent A*1 Rf	0.30	0.30	0.35
B*2	0.46	0.46	0.60
Amino acid analysis (minutes) Rt	43	43	34
Anal	Calcd for $C_5H_9NO_3$:		
	C 45.80	45.68	
	H 6.92	6.71	
	N 10.68	10.34	

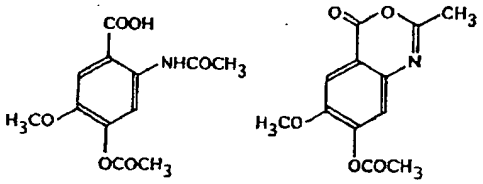
*1 Solvent A: Phenol - H_2O (4: 1)
*2 Solvent B: 10% $AcONH_4$ - acetone - conc NH_4OH (9: 10: 1)

III was heated under reflux with 6 N HCl for 20 hours. The hydrolysate was charged on a column of Dowex 50WX4 which was developed with 0.02 N HCl. The eluted amino acid (IV) was identified as *cis*-4-hydroxy-L-proline by physico-chemical data and co-chromatography. It was differentiated from the *trans* isomer (Table 3). The IR and ^1H NMR spectra of IV were superimposable with those of an authentic sample.

Alkaline Hydrolysis of Chicamycin A (Ia)

The aromatic ring moiety of chicamycin A was not isolated in the above described acid hydrolysis, presumably due to acid instability of the fragment. When Ia was hydrolyzed in 1.0 N NaOH under a nitrogen atmosphere, the reaction mixture contained 4-hydroxy-5-methoxyanthranilic acid[7] as indicated by TLC, though the acid was unstable and could not be isolated as a pure solid. The reaction mixture was, therefore, acetylated *in situ* in pyridine and the product purified by silica gel chromatography to yield two acetyl derivatives, V (minor) and VI (major). The ^1H NMR and MS (M^+ *m/z* 267) revealed that V was 4-acetoxy-5-methoxy-*N*-acetylanthranilic acid. The major product VI was crystallized as colorless needles from methanol. The molecular formula of $C_{12}H_{11}NO_6$ was assigned to VI by MS spectrum (M^+ *m/z* 249) and microanalysis. The IR spectrum of VI lacked the amide band at 1690 cm^{-1} which was present in V. VI is, therefore, 7-acetoxy-6-methoxy-2-methyl-4*H*-3,1-benzoxazin-4-one formed by cyclization of diacetate V (Fig. 4).

Fig. 4. Alkaline degradation products from chicamycin A.



Structures of Chicamycins A (Ia) and B (Ib)

The above hydrolytic results provided confirmation of the structure of Ia as deduced by the ^1H NMR analysis. The stereochemistry at 2 and 11a were established both as *S*-configuration since *cis*-4-hydroxy-L-proline was isolated by the acid hydrolysis of Ia. As stated before, the lack of splitting between H_{11} and H_{11a} in the ^1H NMR of Ia supported an *R*-configuration for C_{11}. Thus, the structure of Ia was assigned as 2(*S*),11(*R*),11a(*S*)-1,2,3,10,11,11a-hexahydro-2,8-dihydroxy-7,11-dimethoxy-5*H*-pyrrolo-[2,1-c][1,4]-benzodiazepin-5-one. Ib has been determined as a demethanol derivative of Ia. The ^1H NMR

of **Ib** indicated an azomethine structure (–N=CH–) leading to the assignment that **Ib** is 2(*S*),11a(*S*)-1,2,3,11a-tetrahydro-2,8-dihydroxy-7-methoxy-5*H*-pyrrolo-[2,1-c][1,4]-benzodiazepin-5-one.

Discussion

Chicamycin is a new member of the pyrrolobenzodiazepine family of antibiotics. As often observed in this group of antibiotics, chicamycin was isolated as either the natural azomethine form (chicamycin B) or its methanol adduct form (chicamycin A). The 1,4-benzodiazepine group of antibiotics may be further divided into 3 subgroups by the substitution pattern on the benzene ring: namely (1) the anthramycin-mazethramycin group, (2) the tomaymycin-neothramycin group and (3) the sibiromycin group. Chicamycin is closely related to neothramycin differing only in the position of a hydroxyl group on the pyrrolidine ring. Neothramycin has a hydroxyl group at the C-3 forming a carbinol amine structure there, whereas chicamycin possesses an α-hydroxyl group at C-2. The greater stability of chicamycin than neothramycin might partly be attributed to the absence of a carbinol amine structure in the pyrrolidine ring. The C_2-β-hydroxyl analog of chicamycin has been synthesized by scientists of Fujisawa[10]. Recently, KANEKO *et al.* of Bristol-Myers prepared the α- and β-hydroxyl isomers of chicamycin and compared their activity (personal communication: A new process for the synthesis of pyrrolo[1,4]-benzodiazepine antitumor antibiotics). It is interesting that the natural α-analog showed higher activity than the corresponding β-form.

Experimental

Acetylation of Chicamycins A (Ia) and B (Ib) to II

A solution of **Ia** (500 mg) in acetic anhydride (2 ml) and pyridine (3 ml) was stirred at room temperature for 4 hours and the mixture was concentrated *in vacuo* to dryness. The residue was dissolved in 1 ml of ethyl acetate and applied on a column of silica gel (ϕ 1.0×50 cm) which was developed with ethyl acetate. Upon monitoring by TLC with solvent system of EtOAc - MeOH (4: 1), the appropriate fractions were pooled and evaporated *in vacuo* to afford 508 mg of diacetyldemethanolchicamycin A (**II**) as white powder. Mp 110~112°C. TLC (EtOAc - MeOH, 4: 1) Rf 0.52. UV λ_{max}^{MeOH} 220 nm (ε 27,000) 243 (sh, 16,200), 320 (3,800). IR ν_{max}^{KBr} 1765, 1738, 1628 cm^{-1}. ^{1}H NMR $\delta_{TMS}^{CDCl_3}$ ppm 2.10 (3H, s), 2.32 (3H, s), 2.4~2.6 (2H, m), 3.5~4.2 (2H, m), 3.88 (3H, s), 5.3~5.5 (2H, m), 7.01 (1H, s), 7.55 (1H, s), 7.82 (1H, d). MS *m/z* 346 (M^+), 304, 286, 244 *etc.*

Anal Calcd for $C_{17}H_{18}N_2O_6 \cdot H_2O$: C 56.04, H 5.53, N 7.69.
Found: C 56.87, H 5.33, N 7.31.

Chicamycin B (**Ib**, 450 mg) was acetylated by an analogous procedure to yield 313 mg of acetate which was identical with **II** by physico-chemical properties.

Oxidation of Diacetyldemethanolchicamycin A (II)

A solution of *m*-chloroperbenzoic acid (900 mg) in 5 ml of CH_2Cl_2 was added dropwise into a solution of **II** (1.39 g) in 5 ml of CH_2Cl_2 at −20°C under vigorous stirring. The mixture was stirred for 3 hours at −20°C, then warmed up to room temperature and filtered. After being washed with saturated $NaHCO_3$ solution to remove acidic products, the solution was evaporated *in vacuo* to a sticky solid which showed a major spot at Rf 0.54 on TLC (EtOAc - MeOH, 4: 1, UV irradiation). The solid was charged on a column of silica gel (ϕ 3.0×40 cm) which was developed with EtOAc. The appropriate fractions were pooled and concentrated *in vacuo* to afford 470 mg of oxo-compound **III**. Mp 130~132°C. TLC (EtOAc - MeOH, 4: 1) Rf 0.54. UV λ_{max}^{MeOH} 228 nm (ε 23,500), 257 (sh, 10,100), 307 (3,600). IR ν_{max}^{KBr} 1770, 1740, 1700, 1635, 1615 cm^{-1}. ^{1}H NMR $\delta_{TMS}^{CDCl_3}$ ppm 1.95 (3H, s), 2.0~2.5 (2H, m), 2.32 (3H, s), 3.85 (3H, s), 3.7~4.2 (2H, m), 5.25 (2H, m), 6.75 (1H, s), 7.48 (1H, s), 9.07 (1H, s). MS *m/z* 362 (M^+), 320, 302, 260, 242 *etc.*

Anal Calcd for $C_{17}H_{18}N_2O_7 \cdot \frac{1}{2}H_2O$: C 54.97, H 5.16, N 7.54.
Found: C 54.50, H 4.91, N 7.20.

Acid Hydrolysis of Oxo-compound (III)

Compound III (470 mg) was heated with 2 ml of 6 N HCl at 105°C for 20 hours in a sealed tube. The hydrolysate was diluted with water and passed through a column of Diaion HP-20 (ϕ 1.0×6 cm) to remove lipophilic products. The spent solution and water washes were combined, decolorized with activated charcoal and concentrated to dryness. The residue was chromatographed on a column of Sephadex G-15 (ϕ 1.0×55 cm) developing with water. The ninhydrin-positive fractions were pooled and concentrated *in vacuo* to give white solid of IV which was crystallized from aqueous ethanol solution, 26.5 mg. Mp 258~259°C (dec). $[\alpha]_D^{24}$ −51.5° (*c* 1.0, H_2O). IR ν_{max}^{KBr} 3200, 2940, 1630, 1570, 1438, 1390 cm^{-1}. ^1H NMR $\delta_{DSS}^{D_2O}$ ppm 2.2~2.6 (2H, m), 3.42 (2H, dd), 4.20 (2H, dd), 4.62 (1H, m). Identified as *cis*-4-hydroxy-L-proline by TLC, ^1H NMR and optical rotation value.

Alkaline Hydrolysis of Chicamycin A (Ia)

A solution of Ia (213 mg) in 20 ml of 1 N NaOH was refluxed for 1 hour under nitrogen atmosphere. The solution was cooled in an ice bath, diluted with 50 ml of water and extracted with two 50 ml-portions of 1-BuOH at pH 5.0. Evaporation of the 1-BuOH extract afforded a sticky solid which contained 4-hydroxy-5-methoxyanthranilic acid by TLC. This solid was acetylated with acetic anhydride (1 ml) and pyridine (3 ml) at room temperature. After addition of 30 ml of water, the reaction mixture was extracted twice with 30 ml of $CHCl_3$. The extracts were combined, dried over anhydrous sodium sulfate and concentrated *in vacuo* to a sticky residue which was chromatographed on a column of silica gel (ϕ 1.0×40 cm). Elution of the column with *n*-hexane - acetone (95: 5) gave the major acetate VI which, upon crystallization from MeOH, afforded 44 mg of colorless needles of VI. Subsequent elution with *n*-hexane - acetone - MeOH (9: 9: 2) afforded the minor acetate V which contained impurities by TLC. This acetate was further chromatographed on a column of silica gel (ϕ 1.0×35 cm) with $CHCl_3$ - AcOH (100: 1) elution to give white powder of V (7.7 mg).

Compound V: Mp 126~127°C. IR ν_{max}^{KBr} 2920, 1760, 1690, 1640, 1520 cm^{-1} *etc.* UV λ_{max}^{MeOH} 226 nm (ε 19,700), 259 (12,000), 316 (4,100). MS *m/z* 267 (M^+), 249, 225, 207, 192, 183 *etc.* ^1H NMR $\delta_{TMS}^{pyridine\text{-}d_5}$ ppm 2.00 (3H, s), 2.18 (3H, s), 3.58 (3H, s), 7.78, (1H, s), 7.86 (NH), 8.65 (1H, s), 12.57 (OH).

Compound VI: Mp 176~178°C. IR ν_{max}^{KBr} 3040, 1775, 1745, 1640, 1500 cm^{-1} *etc.* UV λ_{max}^{MeOH} 231 nm (ε 20,300), 261 (5,500), 277 (sh, 3,000), 318 (2,900), 331 (sh, 2,500). MS *m/z* 249 (M^+), 208, 207, 192 *etc.* ^1H NMR $\delta_{TMS}^{CDCl_3}$ ppm 2.34 (3H, s), 2.43 (3H, s), 3.90 (3H, s), 7.13 (1H, s), 7.55 (1H, s).

Anal Calcd for $C_{12}H_{11}NO_6$: C 57.83, H 4.45, N 5.62.
Found: C 57.65, H 4.41, N 5.53.

Acknowledgment

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EXHIBIT P

BBM-928,* A NEW ANTITUMOR ANTIBIOTIC COMPLEX

III. STRUCTURE DETERMINATION OF BBM-928 A, B AND C

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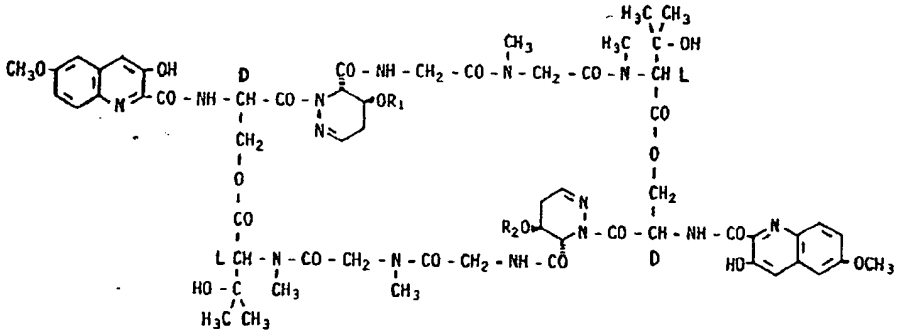
Structures of antitumor antibiotics BBM-928 A, B and C have been determined. They are cyclic decadepsipeptides containing 3-hydroxy-6-methoxyquinaldic acid as a chromophore. Two amino acids, not found in nature, L-β-hydroxy-N-methylvaline and *trans*-(3*S*,4*S*)-4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid, were identified as structural constituents of the antibiotic. In gross structure, BBM-928 resembles the echinomycin group of antibiotics which are cyclic octadepsipeptides having a quinoxaline chromophore, but BBM-928 differs from the latter group by virtue of the lack of a sulfur-containing cross linkage.

BBM-928 is a complex of potent antitumor antibiotics elaborated by a strain of *Actinomadura luzonensis*[1]. The production, isolation, characterization and antitumor activity of BBM-928 have been described in a preceding paper[2]. This paper presents evidence to show that BBM-928 A, B and C possess the following structures:



	R_1	R_2
BBM-928 A	$-COCH_3$	$-COCH_3$
B	$-COCH_3$	H
C	H	H

General Structural Feature and Preliminary Degradation Studies

BBM-928 A, B and C were isolated as colorless crystals and showed physicochemical and spectroscopic properties similar to each other. Nearly identical UV spectra of BBM-928 A, B and C (λ_{max}^{EtOH} at 235, 264 and 345 nm) suggested the same chromophore structure was present in the three components. Acetylation experiments[2] indicated that BBM-928 A and B were, respectively, diacetyl and monoacetyl derivatives of BBM-928 C. Molecular formulae of $C_{64}H_{78}N_{14}O_{24}$, $C_{62}H_{76}N_{14}O_{23}$ and $C_{60}H_{74}N_{14}O_{22}$ were assigned to BBM-928 A, B and C, respectively, based on the results of microanalysis and osmometric molecular weight determination. The molecular weight of BBM-928 A was unequivocally established

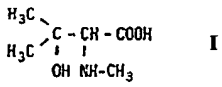
* This antibiotic has recently been named as luzopeptin.

to be 1,426 by field desorption mass spectrometry. The ^{13}C-NMR (CMR) spectrum of BBM-928 A indicated a total of 32 well-defined carbon signals suggesting that the antibiotic consisted of two equivalent halves constituting a sterically symmetric structure. The proton NMR (PMR) supported this assumption, showing the presence of a multiple of nearly 40 protons in the spectrum of BBM-928 A. The PMR and CMR spectra of BBM-928 C also displayed a symmetric structure, whereas those of BBM-928 B indicated an asymmetric conformation.

BBM-928 C was hydrolyzed with 6 N HCl at 110°C for 18 hours in a sealed tube. Lipophilic UV-absorbing material was extracted with *n*-butanol from the hydrolyzate. Amino acid fragments remaining in the aqueous solution were chromatographed on a column of Dowex 50W×4 which was developed by pyridine-formic acid buffer. Four amino acids (I, II, III and IV) eluted in that order were isolated and crystallized from aqueous ethanol. Compound I is a new amino acid whose structure will be discussed later, while II, III and IV were identified as D-serine, sarcosine and glycine, respectively. The solvent-extractable, UV-absorbing fraction was chromatographed on a silica gel column, yielding two chromophoric compounds, V and VI.

Structure of I

Molecular formula of $C_6H_{13}NO_3$ was assigned to I by microanalysis and mass spectrometry ($M^+ + 1$: *m/z* 148). The PMR and CMR of I indicated two C-CH_3, one N-CH_3, one OH, one COOH, one –CH< and one >C<. The pKa′ values (2.3 and 9.5) suggested an α-amino acid structure for I. The presence of a tertiary hydroxyl group was indicated by IR (ν_{OH} 1,160 cm^{-1}) and its reluctance to form acetate. From the above analytical and spectral data, I was determined to be β-hydroxy-N-methylvaline. L-Configuration was assigned to I based on the clear positive COTTON effects observed in its ORD ($[\phi]_{228.5}$ +2,420 (peak))[3] and CD curves ($[\theta]_{208}$ +3,503 (peak))[1]. Racemic β-hydroxy-N-methylvaline has been synthesized by IZUMIYA and NAGAMATSU[5], but the optically active isomer has not been described.



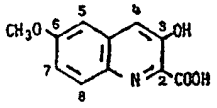
I

Amino acid analysis of the acid hydrolyzate of BBM-928 components indicated that the four amino acids described above (I, II, III and IV) were present in equimolar ratio.

Chromophore Structure of BBM-928

Two UV-absorbing fragments, V and VI, extracted from the acid hydrolyzate of BBM-928 C were acidic compounds and isolated as yellow needles. V was analyzed as $C_{11}H_9NO_4$ and its mass spectrum (MS) showed ion peaks at 219 (M^+), 201 ($M^+ - H_2O$) and 175 ($M^+ - CO_2$, base peak). The presence of a phenolic hydroxyl group in V was shown by positive $FeCl_3$ reaction. The PMR spectrum of V indicated one O-methyl group and four ring protons, and the CMR spectrum included one methyl, one carbonyl and nine aromatic carbons (Table 1). The above information deduced a quinoline or isoquinoline structure for V substituted with one member each of –OH, –OCH_3 and –COOH groups. The chemical shifts of the ring protons (δ 6.89—7.66 ppm) indicated that they were not located vicinal to nitrogen, and hence the 2-position of quinoline or the 1- and 3-positions of isoquinoline should be substituted. Upon irradiation of the methyl protons at δ 3.69 ppm, nuclear OVERHAUSER effect (NOE) was observed

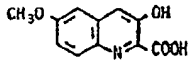
Table 1. PMR and CMR of V.



PMR (60 MHz in D_2O-NaOD)				CMR (in D_2O-NaOD)					
δ (ppm)	M*	J (Hz)	Assignment	δ (ppm)	M*	Assignment	δ (ppm)	M*	Assignment
3.69	s		OCH_3	56.3	q	CH_3	134.5[a]	s	C_{6a}
6.89	d-d	10.5, 1.9	H_7	104.7	d	C_6	155.4	s	C_2
6.91	d	1.9	H_5	116.2	d	C_4	157.5[b]	s	C_3
7.05	s		H_4	118.7	d	C_7	158.4[b]	s	C_8
7.60	d	10.5	H_8	129.0	d	C_8	177.6	s	COOH
				133.2[a]	s	C_{4a}			

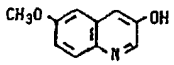
* M: multiplicity; q: quartet; d: doublet; s: singlet.
[a,b] assignment interchangeable.

on two aromatic protons at δ 6.89 and 6.91 ppm (*ca.* 20% enhancement). This observation combined with the multiplicity of ring proton signals indicated that the methoxyl group should be located at the 6-position, and that the 5, 7 and 8 positions were unsubstituted. The CMR spectrum of V showed the presence of only one carbonyl carbon (δ 177.6 ppm, assigned to COOH), indicating that the hydroxyl group of V should be located in the position not allowing tautomerism to a keto form. Thus, a possibility of an isoquinoline structure for V was ruled out, suggesting that the carboxyl and hydroxyl groups should be located at the 2 and 3 positions of the quinoline nucleus, respectively, as shown below:



V

To verify the assigned structure of V, V was decarboxylated to afford VII whose PMR indicated a newly generated H_2 proton at δ 8.36 ppm which coupled with the H_4 proton in the meta position (J= 2.5 Hz).



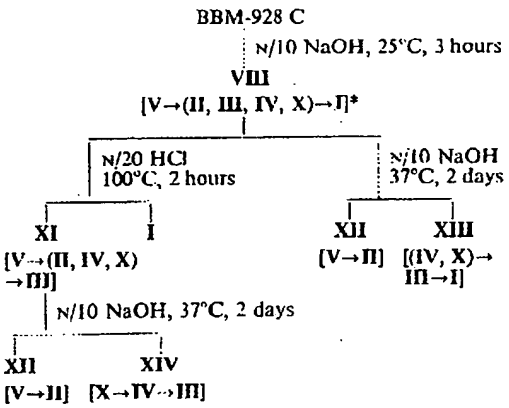
VII

Another chromophoric compound, VI ($C_{10}H_7NO_4$), showed properties similar to those of V except for the absence of methoxy protons in PMR. VI was determined to be 3,6-dihydroxyquinaldic acid, a demethyl derivative of V. VI is considered to be a secondary degradation product formed during drastic acid hydrolysis.

Controlled Hydrolysis Studies (Chart 1)

Upon treatment with 0.1 N NaOH at 25°C for 5 minutes, BBM-928 A and B were quantitatively deacetylated to afford BBM-928 C which was further hydrolyzed under continued stirring for 3 hours to give a new acidic peptide fragment VIII. This compound showed UV and IR spectra similar to those of BBM-928 C except for the absence of ester carbonyl absorption and the appearance of a carboxylate band in the IR spectrum. The presence of free carboxylic acid group in VIII was also indicated by the

Chart 1. Controlled hydrolysis of BBM-928 C.



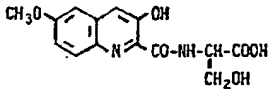
Compound	Structure
I	L-β-Hydroxy-N-methylvaline
II	D-Serine
III	Sarcosine
IV	Glycine
V	3-Hydroxy-6-methoxyquinaldic acid
X	Unknown C_5-unit

* amino acid sequence shown in square bracket

pKa′ determination. Acid hydrolysis of **VIII** in 6 N HCl afforded the same degradation products (**I, II, III, IV, V**) as those obtained from BBM-928 C. I was identified to be the C-terminal amino acid of **VIII** by DAKIN-WEST method. Treatment of **VIII** with excess diazomethane afforded a monomethyl derivative **IX** which was analyzed as $C_{51}H_{41}N_7O_{12}$, the formula being supported by its CMR and MS data.

The above results indicated that BBM-928 C was composed of two moles of **VIII**, which were connected with each other by two ester linkages to form a symmetric cyclic structure. From the analytical and CMR data of BBM-928 C and its degradation products, **VIII** (and accordingly BBM-928 C) should have contained an unidentified moiety of C_5-unit in addition to the five structural constituents (**I**～**V**) described earlier. A molecular formula of $C_5H_8N_2O_3$ was assigned for the C_5-unit which will be designated as compound **X** in the following description.

Since **X** could not be isolated as a single entity because of its presumable instability under the hydrolysis conditions examined above, attempts were made to obtain a small peptide fragment which contained **X** in its intact form. Mild acid hydrolysis of **VIII** (N/20 HCl, 100°C, 2 hours) liberated **I** and afforded a new chromophoric fragment **XI** having **III** in the C-terminal. On the other hand, treatment of **VIII** with 0.1 N NaOH at 37°C for 2 days yielded a UV-absorbing fragment **XII** ($C_{14}H_{14}N_2O_6$) and a non-chromophoric tetrapeptide **XIII**. **XII** consisted of **II** and **V** and its structure was determined to be 3-hydroxy-6-methoxyquinaldyl-D-serine.



XII

A similar alkaline hydrolysis of **XI** also liberated **XII** and afforded a tripeptide fragment **XIV**. The above-described reaction scheme is summarized in Chart 1. Comparative analysis of the PMR and CMR spectra of BBM-928 C, **VIII**, **XIII** and **XIV** indicated that the original structure of **X** remained intact in the small peptide fragments, **XIII** and **XIV**, after the above hydrolytic treatment.

Structure Determination of X (Chart 2)

The C-terminal of tripeptide **XIV** should be **III** as in the parent peptide **XI**. **XIV** did not give an N-dinitrophenyl (DNP) derivative indicating that glycine (**IV**) was not the N-terminal. Therefore the amino

acid sequence in XIV should be X→IV→III. An authentic sample of glycylsarcosine was prepared by a conventional procedure for comparative purposes. Comparison of the CMR of XIV and glycylsarcosine revealed that the following five carbon signals (δ in ppm) were attributable to X: >C=O (173.4), -CH= (140.7), 2×-CH< (61.5 and 61.2) and $-CH_2-$ (30.2). As shown in the PMR of XIV (Fig. 1), irradiation of methylene protons at δ 2.30 ppm converted a triplet at δ 6.72 ppm into a singlet with concomitant simplification of one of the two methine protons, indicating a partial structure of >CH-CH_2-CH= in X. All this structural information led to an assignment of 4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid for the structure of X. In order to verify the proposed structure, XIV was subjected to bromine oxidation to afford an aromatic compound XV ($C_{10}H_{12}N_4O_4$) having UV absorp-

Fig. 1. PMR Spectrum of XIV (60 MHz in D_2O).

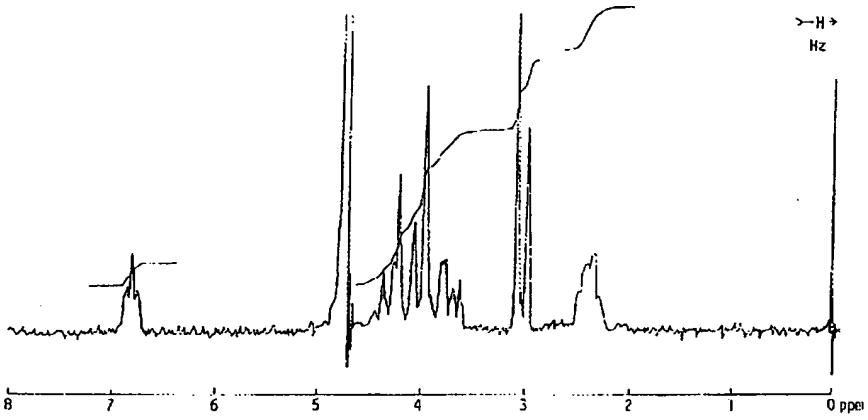
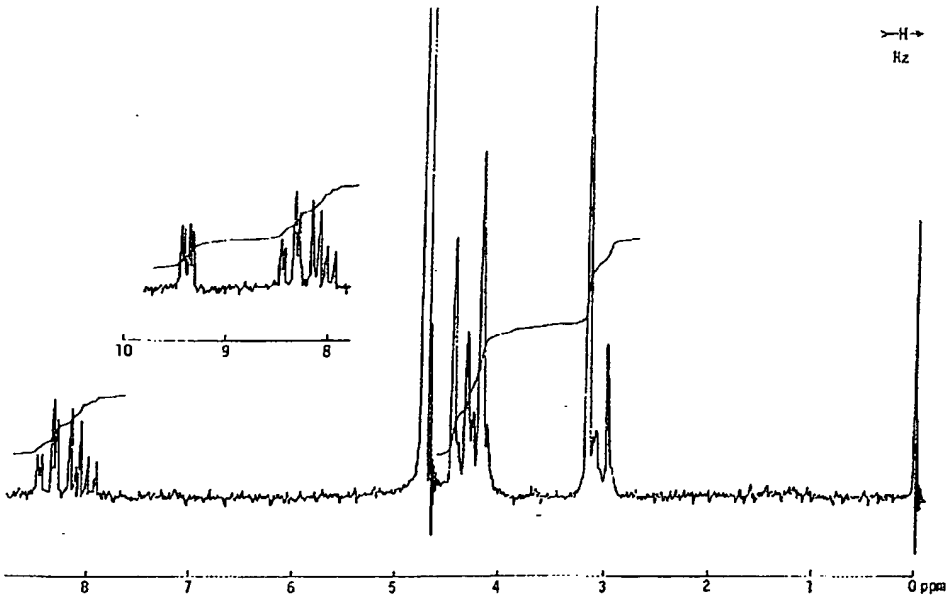


Fig. 2. PMR Spectrum of XV (60 MHz in D_2O).



:osinc was prepared by a
of XIV and glycylsarco-
le to X: >C=O (173.4),
e PMR of XIV (Fig. 1),
into a singlet with con-
structure of >CH-CH$_2$-
y-2,3,4,5-tetrahydropyri-
ised structure, XIV was
$_4$O$_4$) having UV absorp-

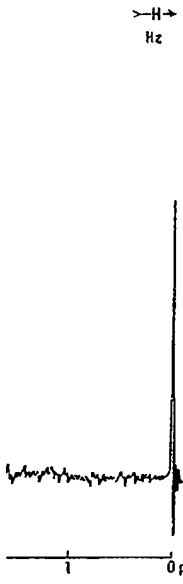
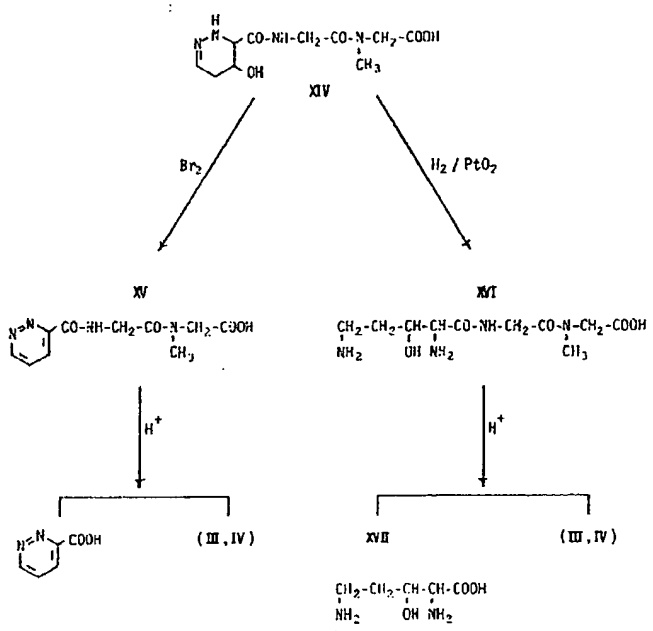
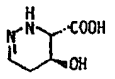


Chart 2. Structures of XIV, XV, XVI and XVII.



tions at 240 and 300 nm. The CMR of **XV** demonstrated the presence of four aromatic carbons and one carbonyl carbon in addition to the signals attributable to **III** and **IV**. As shown in the PMR of **XV** (Fig. 2), the splitting pattern of the aromatic protons indicated a 3-substituted-pyridazine structure. Upon acid hydrolysis, **XV** yielded **III**, **IV** and pyridazine-3-carboxylic acid[6]. The structure of **XV** is therefore 3-pyridazinecarbonylglycylsarcosine.

Catalytic hydrogenation of **XIV** over PtO_2 afforded a new basic peptide **XVI**. Acid hydrolysis of **XVI** in 6 N HCl liberated a new basic amino acid **XVII** ($C_5H_{12}N_2O_4$) along with **III** and **IV**. **XVII** was identified as L-*erythro*-β-hydroxyornithine from its optical rotation value ($[\alpha]_D$ +19.9°) and by direct comparison (TLC, PPC) with a racemic sample[7]. Thus, the structure of **X**, including its stereochemistry, was determined to be *trans*-(3*S*,4*S*)-4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid.

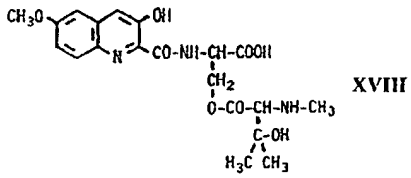


X

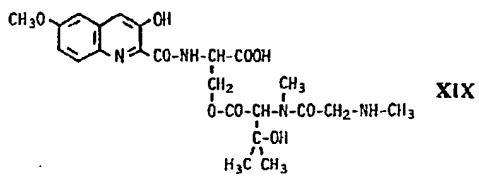
Reductive cleavage of hexahydropyridazine-3-carboxylic acid to ornithine has been described[8]. Structures of **XIV**, **XV**, **XVI** and **XVII** are shown in Chart 2.

Isolation of Depsipeptide Fragments

Under a specific hydrolysis condition (6 N HCl, 100°C, 3 hours), BBM-928 C afforded three solvent-extractable, UV-absorbing fragments, **XII**, **XVIII** ($C_{20}H_{25}N_5O_6$) and **XIX** ($C_{23}H_{30}N_4O_8$), all of which contained the same chromophore moiety, **V**. The IR spectra of **XVIII** and **XIX** indicated the presence of ester carbonyl group ($\nu_{C=O}$ 1,745 cm^{-1}). Upon mild alkaline hydrolysis, **XVIII** yielded **I** and **XII**, while **XIX** afforded **I**, **III** and **XII**. Thus, the structures shown below were assigned to **XVIII** and **XIX**.



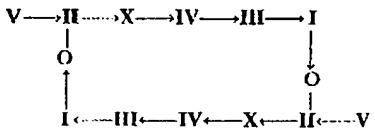
XVIII



XIX

Structure of BBM-928 A, B and C

It has been shown that BBM-928 C is composed of 2 moles of **VIII** cyclized in a symmetrical arrangement and that one mole each of six structural units (**I**, **II**, **III**, **IV**, **V** and **X**) constituted **VIII**. Isolation of peptide fragments, **XI**, **XII**, **XIII** and **XIV**, established the sequence of the six structural constituents in **VIII** as **V**→**II**→**X**→**IV**→**III**→**I**. Since the hydroxyl group of **II** was esterified by **I** in BBM-928 C as revealed by the isolation of depsipeptide fragments **XVIII** and **XIX**, the structure of BBM-928 C was established as that shown below in a schematic form:



BBM-928 A and B were shown to be diacetyl and monoacetyl derivatives of BBM-928 C, respectively. In a comparative PMR analysis, the OH-bearing methine proton of the tetrahydropyridazine moiety (**X**) of BBM-928 C was observed as a broad singlet at δ 4.25 ppm. This proton signal appeared at δ 5.52 ppm in BBM-928 A indicating that the acetylation took place on the hydroxyl group of the tetrahydropyridazine moiety. Thus the structures shown before are assigned to BBM-928 A, B and C.

Discussion

BBM-928 A, B and C are chromophoric cyclic depsipeptides containing 10 amino acid moieties and 2 moles of 3-hydroxy-6-methoxyquinaldic acid, a novel heterocyclic compound. BBM-928 is structurally related to the quinoxaline antibiotics which include echinomycin[9], quinomycins[10] and triostins[11]. The quinoxaline antibiotics are characterized by their cyclic depsipeptide structures which contain 8 amino acids and 2 moles of quinoxaline-2-carboxylic acid as a chromophore. Abundance of N-methyl amino acid moieties as structural constituents is also a common feature of BBM-928 and quinoxaline antibiotics. BBM-928 contains two unusual amino acids, L-β-hydroxy-N-methylvaline (**I**) and *trans*-(3*S*,4*S*)-4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid (**X**), which have not been previously found in nature. In addition to the differences in amino acid and chromophore structures between BBM-928 and quinoxaline antibiotics, BBM-928 does not have a sulfur-containing intramolecular cross linkage which is present in the latter group.

As reported in our previous paper[2], BBM-928 A exhibited the highest antitumor activity among the three components. BBM-928 B, a monoacetyl analog, was about one-third as active as BBM-928 A, while BBM-928 C, a non-acetylated component, was almost devoid of antitumor activity. The PMR spectrum of BBM-928 indicated the presence of unequivalent methylene protons for glycine, sarcosine, serine and tetrahydropyridazine moieties. The difference in chemical shifts for the geminal proton signals was observed to a greater extent in BBM-928 A ($\Delta_{CH_\alpha \cdot H_\beta}$ 0.48~2.07 ppm) than in BBM-928 C ($\Delta_{CH_\alpha \cdot H_\beta}$ 0.13~1.70 ppm). This may reflect a conformational difference in the structures of BBM-928 A and C due to a participation of acetyl groups. The close relationship between biological activity and conformational state has been previously described for cyclic peptide antibiotics[12].

Experimental

Thin-layer chromatography (TLC) was performed on silica gel plate (Kieselgel $60F_{254}$, Merck) using the solvent systems shown below:

System No.	Solvent system	
S-123	MeOH - 10% $AcONH_4$ - 10%NH_4OH	(10: 9: 1)
N-114	*n*-BuOH-Acetone-AcOH-H_2O	(4: 5: 1: 1)
A-107	Phenol-H_2O	(4: 1)
SD-105	$CHCl_3$-*n*-PrOH-10%NH_4OH	(1: 4: 1)
SD-106	$CHCl_3$-MeOH-AcOH	(50: 50: 4)
SD-107	$CHCl_3$-MeOH	(9: 1)

PMR spectra were obtained on a JEOL C60HL or Varian FT80A spectrometer and CMR spectra by Varian FT80A apparatus operated in the FOURIER transform system. Ordinary mass spectra were measured on a Hitachi RMU-6MG mass spectrometer using the direct inlet probe. Amino acid analysis was carried out using a Hitachi 034-2U amino acid autoanalyzer.

Total acid hydrolysis of BBM-928

A solution of BBM-928 C (500 mg) in 20 ml of 6 N HCl was heated at 110°C for 20 hours in a sealed tube. The reaction mixture was diluted with 80 ml of water and extracted with *n*-BuOH (100 ml×2). The aqueous layer was concentrated under reduced pressure to give 415 mg of sticky solid. The solid was chromatographed on a column of Dowex 50W×4 (ϕ 1.5×120 cm) which was pre-equilibrated with 0.1 M pyridine-formic acid buffer (pH 3.1.) The column was developed with the same buffer solution (0.1 M, 480 ml) followed by a 0.2 M buffer (200 ml). The eluate was monitored by ninhydrin test and TLC (S-123). Appropriate fractions were combined and evaporated *in vacuo* to yield I (73 mg), II (37 mg) and III (45 mg) from eluates with 0.1 M buffer solution and IV (39 mg) from 0.2 M buffer eluate.

I: Colorless needles from aqueous EtOH, m.p. 260~261°C (dec.). $[\alpha]_D^c$ −4° (*c* 4.90, 5 N HCl). TLC: Rf 0.72 (S-123) and 0.40 (A-107). PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.22 (3H, s), 1.43 (3H, s), 2.68 (3H, s) and 3.37 (1H, s). CMR $\delta_{DSS}^{D_2O}$ in ppm: 23.9 (q), 28.4 (q), 34.0 (q), 70.7 (s), 73.9 (d) and 171.6 (s). *Anal.* Calcd. for $C_6H_{13}NO_4$: C 48.97, H 8.90, N 9.51. Found: C 48.90, H 9.06, N 9.46. pKa': 2.3 and 9.5 (titration equivalent: 152). CD (*c* 0.05, 0.5 N HCl): $[\theta]_{250}$ 0, $[\theta]_{203}$ +3,503 (peak) and $[\theta]_{201}$ +3,380. ORD (*c* 0.09, 0.5 N HCl): $[\phi]_{300}$ +610, $[\phi]_{220.5}$ +2,420 (peak) and $[\phi]_{220}$ +1,810.

II: Colorless needles from aqueous EtOH, m.p. 222~224°C (dec.). $[\alpha]_D^{22.5}$ −5.3° (*c* 0.8, 5 N HCl). TLC: Rf 0.46 (S-123) and 0.11 (A-107). Identified as D-serine by IR and TLC.

III: Colorless needles from aqueous EtOH, m.p. 208~210°C (dec.). TLC: Rf 0.45 (S-123) and 0.34 (A-107). MS: *m/z* 89 (M^+), 46, 44. *Anal.* Calcd. for $C_3H_7NO_2$: C 40.44, H 7.92, N 15.92. Found: C 39.90, H 8.21, N 15.68. Identified as sarcosine by IR and TLC.

IV: Colorless powder. TLC: Rf 0.49 (S-123) and 0.16 (A-107). Identified as glycine by IR and TLC.

The *n*-BuOH extracts described above were evaporated under reduced pressure to afford 150 mg of yellowish residue which was chromatographed on a column of silica gel (ϕ 2.1×50 cm). The column was developed with a mixture of $CHCl_3$ - *n*-PrOH - 10% NH_4OH, the elution being monitored by absorption at 345 nm. Appropriate fractions were concentrated *in vacuo* to give V (68 mg) from eluate with $CHCl_3$ - *n*-PrOH - 10% NH_4OH (6: 4: 0.2) and VI (35 mg) from eluate of the same solvent system with a ratio of 2: 8: 0.2.

V: Yellowish needles from MeOH, m.p. 223~225°C. TLC: Rf 0.43 (SD-105). λ_{max}^{MeOH} 225 nm (ε 33,100), 256 (30,200) and 350 (12,700). MS: *m/z* 219 (M^+), 201, 175, 173, 147, 132. PMR $\delta_{DSS}^{D_2O+NaOD}$ in ppm: 3.69 (3H, s), 6.89 (1H, d-d, *J*=10.5 & 1.9 Hz), 6.91 (1H, d, *J*=1.9), 7.05 (1H, s) and 7.60 (1H, d, *J*=10.5 Hz). Positive NOE (*ca.* 20%) observed on two protons at δ 6.89 and 6.91 upon irradiation at δ 3.69. *Anal.* Calcd. for $C_{11}H_9NO_4$: C 60.27, H 4.14, N 6.39. Found: C 60.20, H 4.03, N 6.21.

VI: Yellow needles, m.p. 255~257°C (dec.). TLC: Rf 0.37 (SD-105). λ_{max}^{MeOH} 226 nm (ε 36,900), 250 (25,400) and 340 (9,500). MS: *m/z* 205 (M^+), 187, 161, 159. PMR $\delta_{DSS}^{D_2O+NaOD}$ in ppm: 6.52 (1H,

d, J=2.0 Hz), 6.76 (1H, d-d, J=10.5 & 2.0 Hz), 6.90 (1H, s) and 7.52 (1H, d, J=10.5 Hz). *Anal.* Calcd. for $C_{10}H_7NO_4$: C 58.54, H 3.44, N 6.83. Found: C 57.69, H 3.83, N 5.76.

6-Methoxy-3-quinolinol (VII)

V (100 mg) was heated at 225~240°C for 1 minute on a metal bath. Brown residue was taken up in 20 ml of MeOH and insoluble material removed by filtration. Evaporation of the filtrate gave pale yellow solid which was chromatographed on a column of silica gel (ϕ 1.5×45 cm). The column was eluted with $CHCl_3$ - MeOH (99: 1) and elution monitored by absorption at 365 nm. UV-absorbing fraction was evaporated *in vacuo* to afford **VII** (58 mg, 6-methoxy-3-quinolinol). Colorless plates from $CHCl_3$ - MeOH. m.p. 215~216°C. TLC: Rf 0.40 (SD-107). λ_{max}^{MeOH}: 224 nm (ε 44,800), 318 (6,800), 324 (6,800) and 333 (10,200). *Anal.* Calcd. for $C_{10}H_9NO_2$: C 68.56, H 5.18, N 8.00. Found: C 68.34, H 5.11, N 7.86.

Mild alkaline hydrolysis of BBM-928 C-Isolation of VIII

A solution of BBM-928 C (5.3 g) in 0.1 N NaOH (260 ml) was stirred at room temperature for 3 hours. The solution was acidified to pH 2.0 with 6 N HCl and extracted with two 200-ml portions of *n*-BuOH. The extracts were combined, washed with water (300 ml) and concentrated *in vacuo*. Yellowish residue was chromatographed on a column of silica gel (ϕ 2.5×40 cm). The column was developed with $CHCl_3$ - *n*-PrOH - 10% NH_4OH (2: 8: 0.4) and the elution monitored by TLC (N-114) and UV absorption at 345 nm. Fractions showing an Rf 0.27 spot were combined and evaporated *in vacuo* to afford **VIII** (4.09 g). λ_{max}^{MeOH} 232 nm (ε 36,400), 260 (26,500) and 345 (10,400). Drastic acid hydrolysis gave **I**, **II**, **III** and **IV**. DAKIN-WEST degradation and subsequent acid hydrolysis did not afford **I**.

A solution of **VIII** (570 mg) in THF (50 ml) was treated with a large excess of diazomethane solution. After evaporation of the solvent, the residue was chromatographed on a column of silica gel (ϕ 3.0×60 cm) to give **IX** (196 mg). TLC: Rf 0.68 (N-114). MW: 681 (osmometry in MeOH). MS: m/z 654 ($M^+ - H_2O - CH_2O$), 614, 512, 442, 408. *Anal.* Calcd. for $C_{31}H_{41}N_7O_{12} \cdot H_2O$: C 51.58, H 6.01, N 13.58. Found: C 52.10, H 5.93, N 12.38.

Mild acid hydrolysis of VIII—Isolation of XI

A solution of **VIII** (4.0 g) in 200 ml of 0.05 N HCl was heated under reflux for 2 hours and the resulting solution was extracted with two 200-ml portions of *n*-BuOH. Evaporation of the extract *in vacuo* gave yellowish solid which was chromatographed on a silica gel column (ϕ 3.0×50 cm). The column was developed with $CHCl_3$ - MeOH - AcOH (100: 6: 2) and the elution monitored by absorption at 345 nm and TLC (N-114). Appropriate fractions were evaporated *in vacuo* to afford yellowish powder of **XI** (2.75 g). TLC: Rf 0.42 (N-114). λ_{max}^{MeOH} 233 (ε 40,700), 260 (28,000) and 345 (11,500). Complete acid hydrolysis gave **II**, **III** and **IV**. **III** was determined to be the C-terminal of **XI** by DAKIN-WEST method.

Aqueous layer of the hydrolyzate was evaporated to give 1.2 g of sticky solid which contained **I**.

Mild alkaline hydrolysis of VIII—Isolation of XII and XIII

A solution of **VIII** (1.35 g) in 130 ml of 0.1 N NaOH was kept standing at 37°C for 2 days. The mixture was neutralized by Amberlite IRC-50 (H^+) and extracted with *n*-BuOH (200 ml×2). Evaporation of the extracts gave yellowish residue which was chromatographed on a Sephadex LH-20 column (ϕ 3.0×50 cm) using MeOH as eluant. UV-absorbing fractions were combined and concentrated to give **XII** (450 mg). The aqueous layer was concentrated and lyophilized to yield amorphous powder of **XIII** (700 mg, monosodium salt). Acid hydrolysis of **XIII** with 6 N HCl gave **I**, **III** and **IV**.

XII: Colorless needles from MeOH, m.p. 189~191°C. TLC: Rf 0.44 (N-114). λ_{max}^{MeOH} 227 nm (ε 34,300), 261 (25,400) and 345 (11,300). MS: m/z 306 (M^+), 288, 261, 202, 173. PMR $\delta_{TMS}^{DMSO\text{-}d_6}$: 3.86 (2H, m), 3.88 (3H, s), 4.53 (1H, m), 7.18 (1H, d, J=2.0 Hz), 7.21 (1H, d-d, J=10.5 & 2.0 Hz), 7.63 (1H, s), 7.84 (1H, d, J=10.5 Hz), 8.98 (NH, lost with D_2O shake). *Anal.* Calcd. for $C_{14}H_{14}N_2O_6$: C 54.90, H 4.61, N 9.15. Found: C 54.86, H 4.54, N 9.18.

XIII: TLC: Rf 0.10 (SD-106). PMR $\delta_{DSS}^{D_2O}$: 1.17 (3H, s), 1.30 (3H, s), 2.31 (2H, m), 3.03 (3H, s), 3.06 (3H, s), 3.6~4.5 (6H, m), 4.78 (1H, s), 6.73 (1H, t). *Anal.* Calcd. for $C_{16}H_{27}N_3O_7 \cdot Na \cdot H_2O$: C 43.53, H 6.39, N 15.86, Na 5.21. Found: C 43.14, H 6.76, N 15.62, Na 4.99.

Mild alkaline hydrolysis of XI—Isolation of XII and XIV

XI (2.57 g) was treated with 0.1 N NaOH at 37°C for 2 days. The hydrolyzate was neutralized and extracted with *n*-BuOH to afford 1.2 g of yellowish solid which contained XII. The aqueous layer was concentrated *in vacuo* to oily residue which was chromatographed on a column of Diaion HP-20 (ϕ 2.0×40 cm). The column was developed with water and the eluate monitored by ninhydrin test. Concentration of ninhydrin-positive fractions gave white amorphous powder of XIV (1.35 g). TLC: Rf 0.28 (SD-106). MS: *m/z* 255 (M^+-OH), 226, 183, 165, 128. PMR $\delta_{DSS}^{D_2O}$ in ppm: 2.30 (2H, m), 3.02 (3H, s), 3.55~4.4 (6H, m), 6.72 (1H, t). Complete acid hydrolysis of XIV gave III and IV.

Preparation of glycylsarcosine

To a stirred solution of N-carbobenzyloxy(Cbz)-glycine (1.04 g) and methyl sarcosinate (698 mg) in 45 ml of CH_2Cl_2, was added dropwise a solution of dicyclohexylcarbodiimide (DCC, 1.135 g) in 15 ml of CH_2Cl_2 at 0°C. The stirring was continued at 5°C for 16 hours and then 0.1 ml of AcOH was added to decompose excess DCC. The reaction mixture was filtered. The filtrate was washed with 0.1 N HCl followed by saturated $NaHCO_3$ solution and dried over anhydrous sodium sulfate. Evaporation of the solvent yielded methyl Cbz-glycylsarcosinate as oily solid (1.5 g). A part of the solid (492 mg) was kept standing at 0°C with a mixture of EtOH (3 ml) and 1 N NaOH (3.3 ml). The mixture was washed with EtOAc and the aqueous layer was acidified to pH 2.0 and extracted with EtOAc. The EtOAc extract was concentrated *in vacuo* to leave a white residue which was crystallized from benzene and ether. Cbz-glycylsarcosine, 305 mg, m.p. 106~107°C. *Anal.* Calcd. for $C_{13}H_{16}N_2O_5$: C 55.71, H 5.75, N 9.99. Found: C 56.01, H 5.68, N 9.90. A solution of Cbz-glycylsarcosine (194 mg) in 13 ml of EtOH was hydrogenated with 10% paradium on carbon (80 mg) under one atmospheric hydrogen pressure for 19 hours. The catalyst was removed by filtration and the solution evaporated to give 110 mg of glycylsarcosine. TLC: Rf 0.16 (N-114). MS: *m/z* 146 (M^+), 128, 117, 100, 89, 71, 57. PMR $\delta_{DSS}^{D_2O}$ in ppm: 2.97~3.03 (3H), 3.9~4.0 (4H, m).

Bromine oxidation of XIV—Preparation of XV

To a solution of XIV (298 mg) in 200 ml of $CHCl_3$ and 4 ml of AcOH, was added dropwise 200 mg of bromine at room temperature under vigorous stirring. Stirring was continued for one hour and the precipitate deposited was removed by filtration. The yellow filtrate was concentrated *in vacuo* with an addition of toluene to expedite AcOH removal. Lyophilization of the residue gave pale yellow powder of XV (160 mg). TLC: Rf 0.38 (SD-106). $\lambda_{max}^{H_2O}$ 245 nm (ε 1,610) and 305 (410). MS: *m/z* 253 ($M^+ + 1$), 235, 165, 136, 107, 79. PMR $\delta_{DSS}^{D_2O}$ in ppm: 2.98~3.17 (3H), 4.0~4.5 (4H, m,), 8.04 (1H, d-d, J=8.5 & 5.0 Hz), 8.40 (1H, d-d, 8.5 & 1.7 Hz), 9.37 (1H, d-d, J=5.0 & 1.7 Hz).

Treatment of XV with excess diazomethane in ether afforded monomethyl ester of XV which was purified by silica gel chromatography. Colorless prisms, m.p. 173~173.5°C. MS: *m/z* 266 (M^+), 235, 164, 136, 107. λ_{max}^{MeOH} 245 nm (ε 2,100), 310 (160). *Anal.* Calcd. for $C_{11}H_{11}N_3O_4$: C 49.62, H 5.30, N 21.04. Found: C 49.82, H 5.04, N 20.93.

Acid hydrolysis of XV—Isolation of pyridazine-3-carboxylic acid

XV (10 mg) in 1 ml of 6 N HCl was heated at 105~110°C for 18 hours in a sealed tube. The solution was concentrated *in vacuo* to dryness which was triturated with 0.8 ml of water. Insoluble materials were collected by filtration, washed with water and dried *in vacuo* to give 3.1 mg of pyridazine-3-carboxylic acid, m.p. 195~197°C. $\lambda_{max}^{0.1N\ NaOH}$ 250 nm (ε 1,200) and 302 (300). $\lambda_{max}^{0.1N\ HCl}$ 245 (1,400) and 300 (200). IR spectrum identical with that of synthetic sample[6].

Catalytic reduction of XIV—Isolation of XVI

XIV (850 mg) was dissolved in a mixture of AcOH (2 ml), EtOH (3 ml) and water (7 ml), and hydrogenated over PtO_2 at room temperature for 72 hours. The catalyst was filtered off and the filtrate evaporated *in vacuo* to sticky residue. The solid was charged on a column of Amberlite CG-50 (NH_4^+, ϕ 3.0×50 cm) which was developed with water. The elution was followed by ninhydrin test and TLC (S-123). Appropriate fractions were collected and concentrated to afford 280 mg of XVI. TLC: Rf 0.41 (S-123). PMR $\delta_{DSS}^{D_2O+DCl}$ in ppm: 2.0 (2H, m), 3.06 (3H, s), 3.17 (2H, t), 3.55 (1H, d), 3.8~4.3 (5H, m).

Acid hydrolysis of XVI--Isolation of XVII

XVI (180 mg) in 10 ml of 6 N HCl was heated at 105°C for 16 hours in a sealed vessel. Evaporation of the reaction mixture yielded 210 mg of sticky solid which was chromatographed on a column of Amberlite CG-50 (70% NH_4^+, ϕ 1.2×25 cm). The column was first developed with 200 ml of water and then 0.3 N NH_4OH solution. The elution was monitored by ninhydrin test and TLC (S-123). After neutral fragments were eluted with water, XVII was eluted with 0.3 N NH_4OH. Appropriate fractions were concentrated *in vacuo* to yield white powder (96 mg) which was crystallized from aqueous HCl - EtOH to give colorless fine needles of XVII (71 mg). m. p. 255~256°C (dec.). $[\alpha]_D^{24}$ +19.9° (*c* 0.48, 2 N HCl). TLC: Rf 0.26 (S-123). PPC: Rf 0.26 (*n*-BuOH - MEK - c.NH_4OH - H_2O, 5: 3: 3: 1). MS: *m/z* 149 (M^++1), 130, 102, 86, 74. PMR $\delta_{DSS}^{D_2O}$ in ppm: 1.95 (2H, m), 3.17 (2H, t, J=7.3 Hz), 3.84 (1H, d, J=4.3 Hz), 4.22 (1H, m). *Anal.* Calcd. for $C_5H_{12}N_2O_3 \cdot HCl$: C 32.51, H 7.10, N 15.17, Cl 19.22. Found: C 32.59, H 7.11, N 14.93, Cl 18.41. Identified as *erythro*-β-hydroxyornithine by PMR and PPC comparison with authentic sample[7].

Isolation of XII, XVIII and XIX

A solution of BBM-928 C (500 mg) in 50 ml of 6 N HCl was refluxed for 3 hours. The reaction mixture was diluted with 50 ml of water and extracted with three 100-ml portions of *n*-BuOH. The extracts were combined, washed with water and evaporated *in vacuo*. The yellow residue was chromatographed on a column of Sephadex LH-20 (ϕ 3.0×50 cm) using MeOH as eluant. The eluate collected in 2-ml fractions was assayed by UV absorption (at 345 nm) and TLC (N-114). Fractions No. 60~74 which showed an Rf 0.40 spot by TLC were evaporated *in vacuo* to afford XVIII (46 mg). Concentration of fractions No. 108~155 gave XIX (23 mg) and fractions No. 207~220 yielded XII (35 mg). Complete acid hydrolysis of XIX gave amino acids I, II and III.

XVIII: Colorless needles from MeOH, m.p. 242°C. TLC: Rf 0.40 (N-114). λ_{max}^{MeOH} 229 nm (ε 42,000), 261 (28,300) and 345 (12,600). IR: ν_{max}^{KBr} 3370, 1745, 1660, 1627, 1235. MS: *m/z* 377 (M^+−58), 302, 288, 202, 173. PMR $\delta_{TMS}^{DMSO-d_6}$ in ppm: 1.07 (3H, s), 1.15 (3H, s), 2.32 (3H, s), 3.27 (1H, s), 3.87 (3H, s), 4.4~4.8 (3H, m), 7.17 (1H, d), 7.19 (1H, d-d), 7.60 (1H, s), 7.78 (1H, d), 9.24 (NH). *Anal.* Calcd. for $C_{20}H_{25}N_3O_8 \cdot H_2O$: C 52.97, H 6.00, N 9.26. Found: C 53.32, H 5.90, N 9.27.

XIX: Amorphous pale yellow powder. TLC: Rf 0.10 (N-114). λ_{max}^{MeOH} 224 nm (ε 35,800), 262 (26,900) and 345 (10,700). IR: $\nu_{C=O}^{KBr}$ 1740 & 1660.

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